Exhibit L

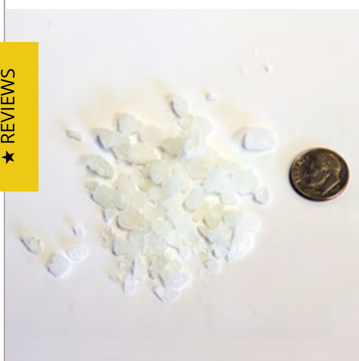
We are currently shipping orders, but our retail showroom remains closed. More on our current policies here. (https://www.talasonline.com/covid-19)

Conservation, Archival & Bookbinding Supplies (/)

Aquazol

★★★☆☆ 1 Review

★ REVIEWS



SELECT WEIGHT :

1/4 Pound | 1/2 Pound | 1 Pound

SELECT FORM :

500 | 200 | 50

SKU: Aquazol

$21.50 to $59.00

QUANTITY

- 1 +

Add to Cart

Add to Wishlist

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Description

Technical Information

Aquazol's excellent water solubility and thermal stability makes it a preferred substitute for PVOH and PVP in high temperature applications. Currently, it is used in a variety of hot-melt and pressure-sensitive adhesive products. and has found its way into conservation as a non-ionic polymer.

Solubility: Highly soluble in many polar, organic solvents such as: acetone, dimethyl formamide, ethanol, methanol, methylene chloride, methyl ethyl ketone, and water. Stable in weak acids and weak bases. Amide group hydrolyzes in strong acids and strong bases.

Physical Data:
*Glass Temperature: 69°C - 71°C (amorphous)
* Refractive Index: 1.52
* Degradation Onset: > 380C (TGA in air)
* Appearance: Light Yellow Solid
* Specific Gravity: 1.14
* Solubility in Water: Freely Soluble
* pH in Aqueous Solution: Neutral
Aquazol 500 molecular weight: 500,000
Aquazol 200 molecular weight: 200,000
quazol 50 molecular weight: 50,000
hemical name: Poly(2-ethyl-2-oxazoline)
rmula: (C5H9NO)

★ REVIEWS

MSDS (/images/PDF/MSDS/aquazal5-50-msds.pdf)

Related Items


(/Glu-Bot)
Glue-Bot (/Glu-Bot)
$4.85
★★★★☆ 6 Reviews


(/Casselli-Spatulas)
Casselli Spatulas (/Casselli-Spatulas)
$20.00
★★★★☆ 8 Reviews


(/Microspatula)
Micro Spatula (/Microspatula)
$3.50
★★★★★ 6 Reviews


(/Olfa-Cutter-SVR-1)
Olfa Cutter SVR-1 (/Olfa-Cutter-SVR-1)
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yotpo%2F&utm_campaign=branding_link_reviews_widget_v2&utm_medium=widget&u

★★★☆☆ 3.0
1 Review
0 Questions \ 0 Answers

★★★★★ (0)
★★★★☆ (0)
★★★☆☆ (1)
★★☆☆☆ (0)
★☆☆☆☆ (0)

WRITE A REVIEW

ASK A QUESTION

Reviews (1) Questions (0)

Annick T. Verified Buyer 07/03/20
★★★☆☆
Great but...

Received my package as expected expect that I assume it wasn't well scraped and as a result the glue granules melted together because of the relative humidity and now the entire package is impossible to separate.

Share |

Was This Review Helpful? 0 0

Reviewed on: Aquazol (https://yotpo.com/go/n5MdQkfR)

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(/A uazol)

zol (/Aquazol)

★ REVIEWS

50

★★★☆☆
1 Review

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330 Morgan Ave. Brooklyn, NY 11211 212-219-0770

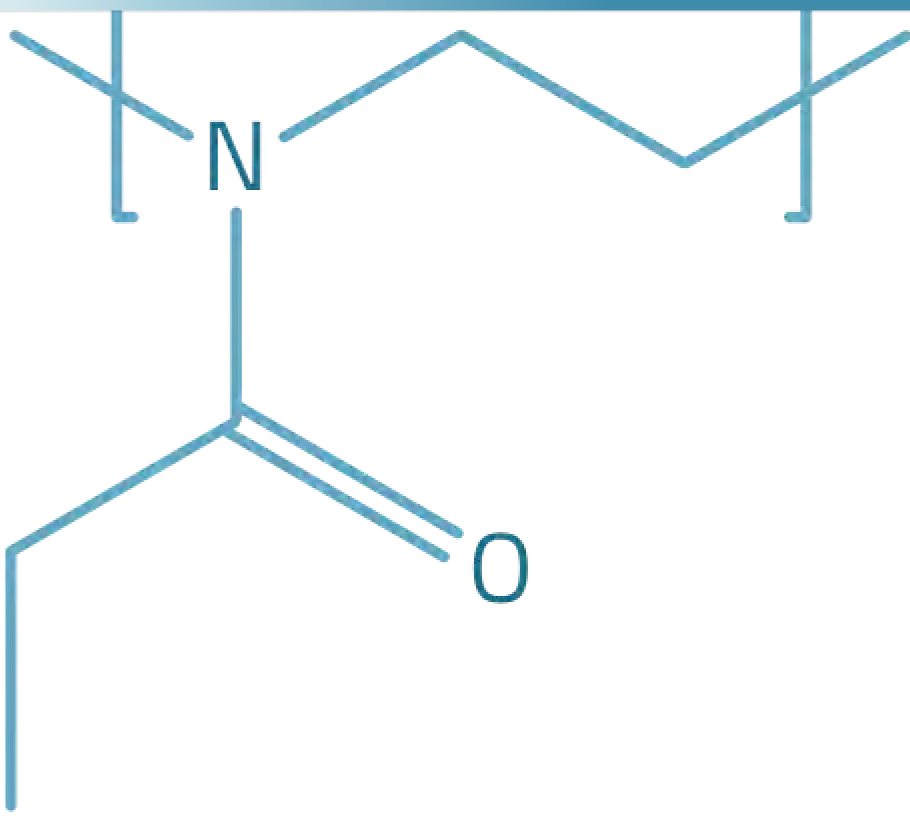
Aquazol®

Poly(2-ethyl-2-oxazoline)

A High Performance Water-Soluble Polymer

Aquazol, poly(2-ethyl-2-oxazoline) – also known as PEOX or PEtOx, is a unique and innovative water-soluble polymer which is non-toxic, non-hazardous, and biocompatible. Among other novel characteristics, this polymer is temperature responsive and inversely soluble above its LCST, ~66°C.

Polymer Chemistry Innovations, Inc.



Aquazol's numerous advantages come from its unique design, featuring a stable amide group (found in natural proteins), with the amide nitrogen in the backbone of the polymer and the amide carbonyl group in the dangling side chain. The doubly bonded, electronegative oxygen in the carbonyl is structurally positioned to readily hydrogen-bond. This novel and innovative design of the polymer backbone presents many advantages over other in-class polymers (PVA, PEG, PVP), including:

- Increased Stability under Temperature, Shear, Oxidation, and UV
- Unparalleled "Actives" Loading Capability
- Incredibly Broad Solubility
- Precise Control of Viscosity
- Excellent Film Forming

Polymer Chemistry Innovations, Inc.

- Strong Adhesion
- Impressive Compatibility
- Especially Broad Dispersity
- Ease of Processing

Product Specifications

Aquazol is supplied as 100% active off-white amorphous granules in four different grades, all having a poly-dispersity range of 3 – 4. Aquazol grades are based on kinematic viscosity, as detailed in the table below:

Product Grade	Kinematic Viscosity Range (10% Aqueous Solution)
Aquazol 5	2 – 3 cSt
Aquazol 50	5 – 7 cSt
Aquazol 200	18 – 24 cSt
Aquazol 500	60 – 80 cSt

For Aquazol polymer with a narrow pdi (<1.9) and targeted

Polymer Chemistry Innovations, Inc.

Chemical Specifications

Chemical Name

Poly(2-ethyl-2-oxazoline)

Formula

$-(C_5H_9NO)_n-$

CAS Registry Number

25805-17-8

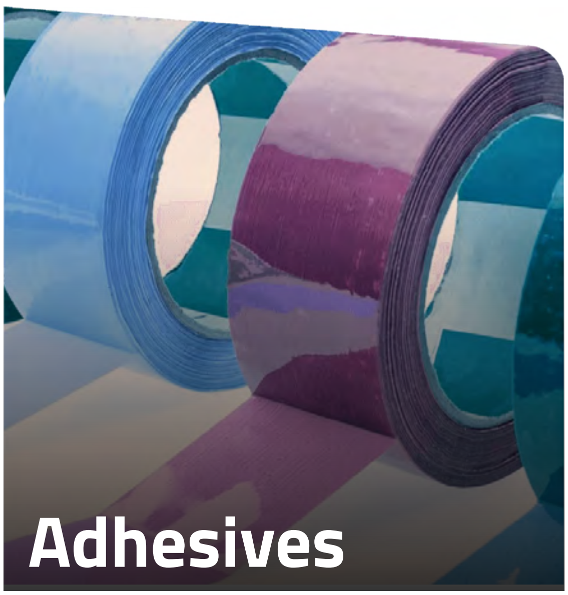
Physical Properties

Property	Result
Appearance	Off-white solid
Refractive Index	1.52
Glass Temperature	69°C
Degradation Onset	380°C in air
Vicat Softening Temperature	70°C
Cloud Point (5% w/w)	68°C
Specific Gravity	1.14
Dielectric Constant	3.68
Solubility Parameter	25.73
Solubility in Water	Freely Soluble
pH in Aqueous Solution	Neutral

Polymer Chemistry Innovations, Inc.

Industrial Application

Aquazol is available in four different grades, each specifically targeted to provide customers with a different set of attributes for their products. Among other differences, the viscosity varies greatly at the different grades, providing specialized performance in a wide range of industries.



Polymer Chemistry
Innovations, Inc.

SEE ALL INDUSTRIES

Regulatory Information

- Listed on US TSCA Inventory
- Approved by FDA for use as an indirect food additive (adhesive) under 21 CFR 175.105
- Not found to be in any hazard category defined by SARA Title III, Sections 311 and 312

Polymer Chemistry Innovations, Inc.

a binder in disinfectant with residual activity on a hard, non-porous surface

- RoHS (2011/65/EU) Compliant

Technical Information

To find the information necessary for your specific application in printable format, please visit our substantial ***library of technical data sheets*** or view our ***FAQ***. Otherwise, please do not hesitate to ***contact us via our online form***. We enjoy answering questions!

Aquazol Safety Data Sheet

Polymer Chemistry Innovations, Inc.

LOCATIONS

Polymer Chemistry Innovations, Inc.
4231 S. Fremont Avenue
Tucson, AZ 85714 USA

(520) 746-8446

Polymer Chemistry Chocolate Bayou, LLC.
7302 County Road 171
Alvin, TX 77511 USA

(520) 746-8446

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LOCATIONS

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4231 S. Fremont Avenue
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(520) 746-8876

Polymer Chemistry Chocolate Bayou, LLC.
7302 County Road 171
Alvin, TX 77511 USA

(520) 746-8876

SIGMA-ALDRICH

Product Results

Building Blocks Explorer

Shipping

- [ ] Ships Today

Product Category

- [ ] Poly(2-ethyl-2-oxazoline) (3)
- [ ] Well-defined Poly(2-ethyl-2-oxazoline) (1)

Physical Form

Available For Sale

USA
Globally

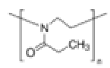
Search term: "25805-17-8"

Compare Products: Select up to 4 products.

4 matches found for 25805-17-8 Advanced Search | Structure Search

Poly(2-ethyl-2-oxazoline)

3 Product Results | Match Criteria: CAS Number, Related Cas Number



Synonym: **PEOX, POx, PetOx, poly 2-ethyloxazoline, polyethyloxazoline**

Linear Formula: **[-N(COC$_2$H$_5$)CH$_2$CH$_2$-]$_n$** | CAS Number: **25805-17-8**

372846 average M$_w$ ~50,000, PDI 3-4 Sigma-Aldrich SDS Close

SKU-Pack Size	Availability	Pack Size	Price (USD)	Quantity
372846-100G	Available to ship on 08/14/20 - FROM	100 g	40.00	0
372846-500G	Available to ship on 08/14/20 - FROM	500 g	127.00	0

Bulk orders? ADD TO CART

373974 average M$_w$ ~500,000, PDI 3-4 Sigma-Aldrich SDS Close

SKU-Pack Size	Availability	Pack Size	Price (USD)	Quantity
373974-100G	Available to ship on 08/14/20 - FROM	100 g	46.30	0
373974-500G	Only 1 left in stock (more on the way) - FROM	500 g	159.00	0

Bulk orders? ADD TO CART

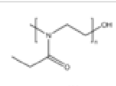
372854 average M$_w$ ~200,000, PDI 3-4 Sigma-Aldrich SDS Close

SKU-Pack Size	Availability	Pack Size	Price (USD)	Quantity
372854-100G	Available to ship on 08/14/20 - FROM	100 g	50.70	0

Bulk orders? ADD TO CART

Ultroxa®: Poly(2-ethyl-2-oxazoline)

1 Product Result | Match Criteria: CAS Number, Related Cas Number



Linear Formula: **CH$_3$(C$_5$H$_9$NO)$_n$OH**

900353 average M$_n$ 50,000, PDI ≤1.25 Sigma-Aldrich SDS Pricing

1

Alfa.com will be undergoing maintenance on Saturday, August 15th and Sunday, August 16th

Feedback

Search Results

Your search results for *poly-2-ethyl-2-oxazoline*

Expand all

Hierarchy Filter

Organic Polymers (3) (/en/search/?q=poly-2-ethyl-2-oxazoline&selected_facets=categories_exact:Organic%20Polymers)

42396 (/en/catalog/042396/) Poly(2-ethyl-2-oxazoline), M.W. 50,000

CAS: 25805-17-8 (/en/cas/25805-17-8/)

[25805-17-8], [-N(COC$_2$H$_5$)CH$_2$CH$_2$-]$_n$, Off-white solid, MDL MFCD00134377, TSCA Yes

Sang Cheon Lee.; Youngkyu Chang.; Jin-San Yoon.; Chulhee Kim.; Ick Chan Kwon.; Yong-Hee Kim and Seo Young Jeong. Synthesis and Micellar Characterization of Amphiphilic Diblock Copolymers Based on Poly(2-ethyl-2-oxazoline) and Aliphatic Polyesters.*Macromolecules.* **1999,** *32*(6), 1847-1852.

Structure image

Stock No.	Size	Price ($)	Quantity	Availability
42396-22	100g	29.10		
42396-36	500g	103.00		

Stock No.	Size	Price ($)	Quantity	Availability
42396-A4	2.5kg	378.00		

Add to Cart | Bulk/Specialty | Print Quote | View Item (/en/catalog/042396/)

42397 (/en/catalog/042397/) Poly(2-ethyl-2-oxazoline), M.W. 200,000

CAS: 25805-17-8 (/en/cas/25805-17-8/)

[25805-17-8], [-N(COC_2H_5)CH_2CH_2-]$_n$, Off-white solid, MDL MFCD00134377, TSCA Yes

Celebi, O.; Barnes, S. R.; Narang, G. S.; Kellogg, D.; Mecham, S. J.; Riffle, J. S. Molecular weight distribution and endgroup functionality of poly(2-ethyl-2-oxazoline) prepolymers. *Polymer* **2015,** *56,* 147-156.

Cataldi, A.; Deflorian, F.; Pegoretti, A. Poly 2-ethyl-2-oxazoline/microcrystalline cellulose composites for cultural heritage conservation: Mechanical characterization in dry and wet state and application as lining adhesives of canvas. *Int. J. Adhes. Adhes.* **2015,** *62,* 92-100.

Structure image

Stock No.	Size	Price ($)	Quantity	Availability
42397-22	100g	27.20		
42397-36	500g	82.30		
42397-A4	2.5kg	327.00		

Add to Cart | Bulk/Specialty | Print Quote | View Item (/en/catalog/042397/)

42398 (/en/catalog/042398/) Poly(2-ethyl-2-oxazoline), M.W. 500,000

CAS: 25805-17-8 (/en/cas/25805-17-8/)

[25805-17-8], [-N(COC_2H_5)CH_2CH_2-]$_n$, Off-white solid, MDL MFCD00134377, TSCA Yes

Sang Cheon Lee.; Youngkyu Chang.; Jin-San Yoon.; Chulhee Kim.; Ick Chan Kwon.; Yong-Hee Kim and Seo Young Jeong. Synthesis and Micellar Characterization of Amphiphilic Diblock Copolymers Based on Poly(2-ethyl-2-oxazoline) and Aliphatic Polyesters.*Macromolecules.***1999,***32*(6), 1847-1852.

Structure image

Feedback

Stock No.	Size	Price ($)	Quantity	Availability
42398-22	100g	28.50		
42398-36	500g	83.70		
42398-A4	2.5kg	342.00		

Add to Cart Bulk/Specialty Print Quote View Item (/en/catalog/042398/)

Feedback

2-(p-Tolyl)-5,5-dimethyl-1,3,2-dioxaborinane, see 4-Methylbenzeneboronic acid neopentyl glycol ester, 99% (/en/search/?q=4-Methylbenzeneboronic%20acid%20neopentyl%20glycol%20ester%2C%2099%25)

« (?q=poly-2-ethyl-2-oxazoline&page=1) ← 1 → » (?q=poly-2-ethyl-2-oxazoline&page=1)

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or 1-978-521-6350
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Poly(2-ethyl-2-oxazoline), Cat# 924

Home → Product → **Poly(2-ethyl-2-oxazoline), Cat# 924**

Poly(2-ethyl-2-oxazoline), Cat# 924

Select Size To View Price

- 50g
- 100g

$56.00

1 + - Add to cart

CAT: 924. Category: Polymers.

Product Information Reviews (0)

Product Information

SDS-Click to download

Size	*50g, 100g*

Physical Form:	Chunks
Approx Mw:	5,000
Density:	1.14
Refractive Index:	1.52
Tg (C):	70
Soluble in:	Acetone, DMF, ethanol, MEK, methanol, methylene chloride, THF, water
TSCA:	TSCA listed
CAS:	25805-17-8

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Poly(2-ethyl-2-oxazoline), Cat# 925

Home → Product → **Poly(2-ethyl-2-oxazoline), Cat# 925**

Poly(2-ethyl-2-oxazoline), Cat# 925

Select Size To View Price

- 50g
- 100g

$35.00

1 Add to cart

CAT: 925. Category: Polymers.

Product Information | Reviews (0)

Product Information

SDS-Click to download

Size	*50g, 100g*

Physical Form:	Granular
Approx Mw:	50,000
Density:	1.14
Refractive Index:	1.52
Tg (C):	70
Soluble in:	Acetone, DMF, ethanol, MEK, methanol, methylene chloride, THF, water
TSCA:	TSCA listed
CAS:	25805-17-8

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Poly(2-ethyl-2-oxazoline), Cat# 926

Home → Product → **Poly(2-ethyl-2-oxazoline), Cat# 926**

Poly(2-ethyl-2-oxazoline), Cat# 926

Select Size To View Price

- 50g
- 100g

$56.00

1 Add to cart

CAT: 926. Category: Polymers.

Product Information | Reviews (0)

Product Information

SDS-Click to download

Size	*50g, 100g*

Physical Form:	Granular
Approx Mw:	200,000
Density:	1.14
Refractive Index:	1.52
Tg (C):	70
Soluble in:	Acetone, DMF, ethanol, MEK, methanol, methylene chloride, THF, water
TSCA:	TSCA listed
CAS:	25805-17-8

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Poly(2-ethyl-2-oxazoline), Cat# 927

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Poly(2-ethyl-2-oxazoline), Cat# 927

Select Size To View Price

- 50g
- 100g

$56.00

1 + - Add to cart

CAT: 927. Category: Polymers.

Product Information Reviews (0)

Product Information

SDS-Click to download

Size	*50g, 100g*

Physical Form:	Granular
Approx Mw:	500,000
Density:	1.14
Refractive Index:	1.52
Tg (C):	70
Soluble in:	Acetone, DMF, ethanol, MEK, methanol, methylene chloride, THF, water
TSCA:	TSCA listed
CAS:	25805-17-8

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Exhibit M

Polysciences, Inc.

Catalog# 24765 – Polyethylenimine HCl MAX, MW 40000, Transfection Grade

Transfection Reagent Preparation and Storage Recommendations

Transfection Reagent Preparation (1 mg/mL)

Materials

- 1g PEI MAX 40K (Polysciences Catalog# 24765)
- 1L Milli-Q® water, water for injection (WFI), or comparable biological-grade water
- 1N Sodium Hydroxide, USP
- Sterile 25 mL plastic pipette
- 0.1µm, 0.2µm, or 0.22µm PES vacuum sterile-filter unit
- Sterile HDPE or polypropylene storage vials

Equipment

- 1L glass beaker
- PTFE-coated stir bar
- Magnetic stir plate
- 1L glass graduated cylinder
- pH meter
- Pipette controller
- Vacuum pump with tubing

Method

1. In 1L glass beaker, suspend 1g of PEI MAX 40K in 900 mL water.

2. Add PTFE-coated stir bar to 1L glass beaker and set stirring to produce small vortex.

3. Wait for PEI MAX 40K to completely dissolve. This typically takes less than 5 minutes.

4. Use 25 mL plastic pipette to add 1N sodium hydroxide dropwise to 1L glass beaker until pH is 6.90 to 7.10.

5. If pH accidently exceeds 7.10, use 1N hydrochloric acid and a new 25 mL plastic pipette to lower pH back to 6.90 to 7.10.

6. Decant solution from 1L glass beaker to 1L graduated cylinder.

7. Add water to 1L graduated cylinder to adjust final volume to 1L.

8. Sterile-filter solution through vacuum membrane.

9. Aliquot solution as desired.

10. Store aliquots at 4°C

Transfection Solution Storage and Shelf Life

At 4°C in a suitable container, the transfection solution will retain its maximum performance for at least six months. For qualitative work, the solution may be suitable for use for up to a year.

Support

We're here to help! Our technical staff can troubleshoot most common and not-so-common issues. Send us an email at **info@polysciences.com** and we will get back to you, typically within one business day.

Pre-made Solutions

We offer pre-made solutions of PEI MAX 40K as our Transporter 5™ Transfection Reagent (Catalog# 26008). Transporter 5™ is 0.1µm sterile-filtered, fully qualified for transfection, and continuously retested to ensure reliably high protein yields.

Exhibit N

Methods Enzymol. Author manuscript; available in PMC 2014 May 7.
Published in final edited form as:
Methods Enzymol. 2013; 529: 227–240.
doi: 10.1016/B978-0-12-418687-3.00018-5
PMCID: PMC4012321
NIHMSID: NIHMS572578
PMID: 24011049

Transient Mammalian Cell Transfection with Polyethylenimine (PEI)

Patti A. Longo, Jennifer M. Kavran, Min-Sung Kim, and Daniel J. Leahy[1]

Johns Hopkins University School of Medicine, Baltimore, MD, USA
[1]Corresponding author: dleahy@jhmi.edu

Abstract

Standard protein expression systems, such as *E. coli*, often fail to produce folded, mono-disperse, or functional eukaryotic proteins (see Small-scale Expression of Proteins in *E. coli*). The expression of these proteins is greatly benefited by using a eukaryotic system, such as mammalian cells, that contains the appropriate folding and posttranslational machinery. Here, we describe methods for both small- and large-scale transient expression in mammalian cells using polyethylenimine (PEI). We find this procedure to be more cost-effective and quicker than the more traditional route of generating stable cell lines. First, optimal transfection conditions are determined on a small-scale, using adherent cells. These conditions are then translated for use in large-scale suspension cultures. For further details on generating stable cell lines please (see Rapid creation of stable mammalian cell lines for regulated expression of proteins using the Gateway® Recombination Cloning Technology and Flp-In T-REx® lines or Generating mammalian stable cell lines by electroporation).

1. THEORY

DNA can be introduced into a host cell by transfection with polyethylenimine (PEI), a stable cationic polymer (Boussif et al., 1995). PEI condenses DNA into positively charged particles that bind to anionic cell surfaces. Consequently, the DNA:PEI complex is endocytosed by the cells and the DNA released into the cytoplasm (Sonawane et al., 2003). Our laboratory uses PEI over other cell transfection reagents because of its low cost.

This protocol is appropriate for two suspension cell lines, CHO-S and HEK *293 GnTi-*. Many cell lines can be transfected successfully with PEI but in our experience these two cell lines express the highest level of protein compared to other cells.

2. EQUIPMENT

Laminar flow hood

CO_2 incubator

Platform shaker

Centrifuge

Water bath (37 °C)

Inverted microscope

Hemacytometer

Sterile 0.22 µm filters

Sterile 250-ml polypropylene centrifuge tubes

Sterile 50-ml polypropylene conical tubes

Sterile 1.5-ml polypropylene tubes

Sterile 6-well tissue culture plates micropipettors

Sterile micropipettor tips

Sterile disposable serological pipettes

Sterile square polypropylene bottles

3. MATERIALS

Plasmid DNA directing your protein of interest

Fetal bovine serum (FBS, Invitrogen)

Polyethylenimine ‘Max’ (linear, MW 25 000) (Polysciences, Inc.)

L-Glutamine 100× (Invitrogen)

Sodium hydroxide (NaOH)

MEM α (containing Earl’s Salts and l-glutamine, but no ribonucleosides, deoxyribonucleosides, $NaCO_3$; Invitrogen 12000)

DMEM/F12 (with L-glutamine, but no HEPES, $NaHCO_3$; Invitrogen 12500)

Freestyle™ 293 medium (Invitrogen 12338-026)

FreeStyle™ CHO-S (Invitrogen R800-07)

Hybridoma SFM (Invitrogen 12045)

Opti-MEM® (Invitrogen)

HEK293S GnTI- (ATCC# CRL-3022)

HEK293T/17 (ATCC# 11268)

Note	*Some of the stock solutions come with the pH indicator phenol red. This supplement does not affect the application and might be useful if the researcher wishes to visualize any pH changes that can occur in the solutions over time. In the case of non-CO_2 incubators (e.g., when scaling-up the production of adherent cells in roller bottles), HEPES-buffered media can be used to keep the pH stable.*
Note	*Catalog numbers are from the US website of Invitrogen and may differ on other local websites.*

3.1. Solutions & buffers

PEI 'Max'		
Dissolve 1 g PEI 'Max' in 900 ml distilled water. Adjust the pH to 7.0 with 1 N NaOH. Add distilled water to 1 l		
Note: Stable at least 9 months at 4 °C		
Make smaller volumes depending on how much is needed. PEI 'Max' cannot be frozen!		
FreeStyle™ 293 'Completed'		
Component	**Stock**	**Amount**
FreeStyle™ 293 medium		1 l
FBS	100%	10 ml
L-Glutamine	200 mM	10 ml
DMEM:F12, 5% FBS		
Add 50 ml FBS to 1 l of DMEM:F12		
Alpha MEM, 5% FBS		
Add 50 ml FBS to 1 l of Alpha MEM		
Hybridoma SFM, 1% FBS		
Add 10 ml FBS to 1 l of Hybridoma SFM		

4. PROTOCOL

4.1. Preparation

Before transfection, sterile high-quality DNA must be prepared. The vector containing the appropriate expression promoter (see Molecular Cloning) and the gene of interest should be transformed into a recA- strain of *E. coli* (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation) and then the plasmid DNA isolated (see Isolation of plasmid DNA from bacteria). Commercially available, endotoxin-free kits for large-scale plasmid DNA isolation produce

sufficiently high-quality DNA. High-quality DNA is characterized as having an OD260/280 ratio between 1.88 and 1.92, an OD260/230 ratio of 2.1–2.2, and a concentration above 0.5 mg ml^{-1} (see Explanatory Chapter: Nucleic Acid Concentration Determination).

Cells must be greater than five passages from liquid nitrogen, adapted to media, free of mycoplasma contamination, and single cells if in suspension culture. All steps are carried out using sterile technique in a laminar flow hood. Solutions should be sterile-filtered through 0.22-µm filters. All plastic and glassware, if not purchased as sterile, should be double autoclaved. Cell growth media should be warmed to 37 °C prior to contact with cells. Different growth media are needed for each cell line and growth condition. These media are listed in Table 18.1. Each time the protocol says to use 'media,' use the appropriate media as outlined in Table 1 for the specific cell line and growth conditions. Cell type used depends on specific needs of protein of interest. In our laboratory, optimized protein expression conditions determined for adherent cells translate well into large-scale suspension conditions for the same cell line.

Table 18.1

Cells: Growth characteristics and medium

Cell line	**Growth type**	**Ideal medium**	**Cell source**
HEK293S GnTI-	Suspension	Freestyle™ 293 'completed'	ATCC# CRL-3022
HEK293S GnTI-	Adherent	DMEM:F12, 5% FBS	ATCC# CRL-3022
HEK293T/17	Adherent	DMEM:F12, 5% FBS	ATCC# CRL-11268
CHO-S	Suspension	Hybridoma SFM, 1% FBS	Invitrogen R800-07
CHO-S	Adherent	DMEM:F12, 5% FBS	Invitrogen R800-07

4.2. Duration

Preparation	1 week
Protocol	1–2 weeks

See Fig. 18.1 for the flowchart of the complete protocol.

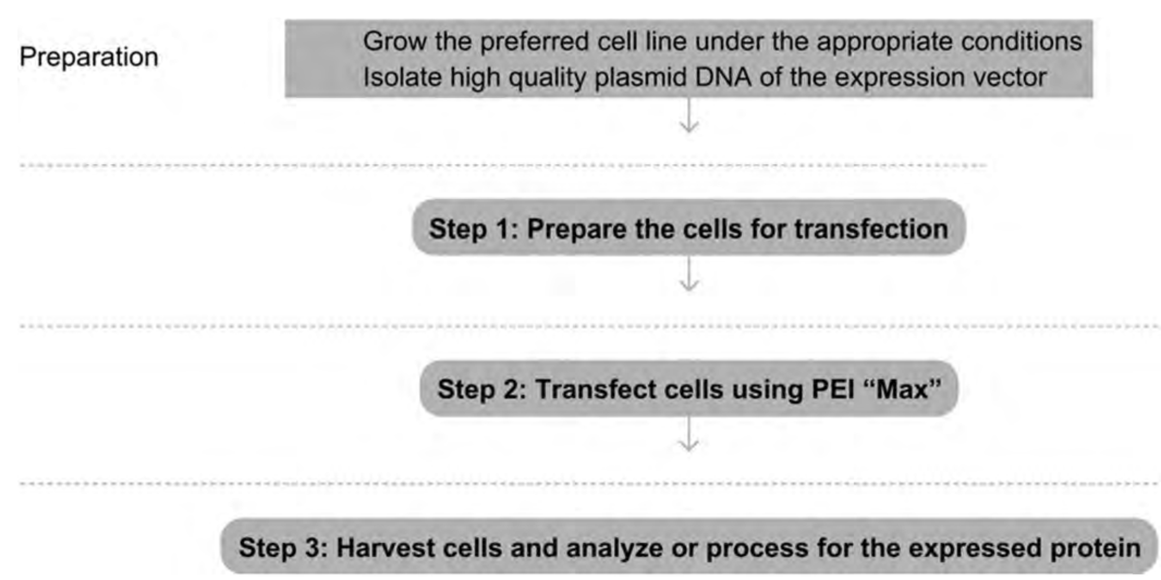

Figure 18.1

Flowchart of the complete protocol, including preparation.

5. STEP 1 SMALL-SCALE TRANSIENT TRANSFECTION

5.1. Overview

This step will prescreen a variety of transfection conditions including media, cell type, ratio of PEI to DNA, and expression time to maximize protein expression before scaling up to a large-scale transfection.

5.2. Duration

5 days

Note *Transfection must be done in the absence of antibiotics.*

6. STEP 1.1 SEED ADHERENT CELLS FOR TRANSFECTION

6.1. Overview

The appropriate amount of cells are transferred to a 6-well dish and allowed to become adherent.

6.2. Duration

1.5 days

1.1.1 Wash the cells with PBS, trypsinize, add 10ml DMEM:F12 + 5% FBS, and gently pipet the cells several times to ensure an even suspension before counting. Transfer the cells to a 15-ml sterile centrifuge tube. Count the cells using a hemacytometer.

1.1.2 For each transfection condition to be tested, aliquot 3×10^5 cells into a sterile centrifuge tube.

1.1.3 Spin the cells at $200 \times g$ at room temperature for 5 min. Aspirate supernatant.

1.1.4 Add DMEM:F12 + 5% FBS to the cell pellet to a final concentration of 3×10^5 cells ml^{-1}.

1.1.5 Resuspend the cells with a serological pipette.

1.1.6 Add 2 ml of DMEM:F12+5% FBS to each well of a 6-well plate.

1.1.7 Transfer 1 ml of the cell suspension to each well.

1.1.8 Place dish in the 37 °C incubator, 5% CO_2.

1.1.9 After 24 h, remove the media from each well.

1.1.10 Add 2.7 ml of fresh DMEM:F12 + 5% FBS to each well.

1.1.11 Return the dish to incubator.

See Fig. 18.2 for the flowchart of Step 1.1.

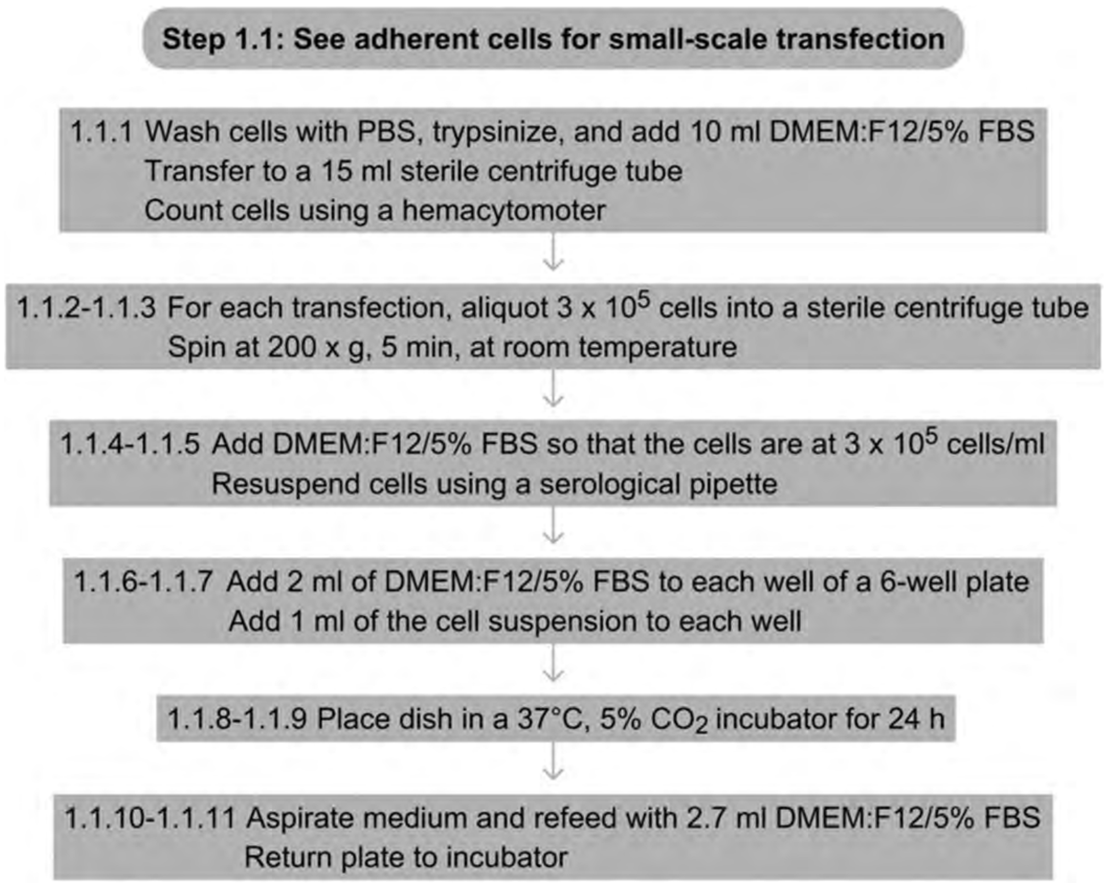

Figure 18.2

Flowchart of Step 1.1.

7. STEP 1.2 TRANSIENTLY TRANSFECT CELLS

7.1. Overview

Cells are transfected by adding DNA and PEI 'Max' to the cells. The PEIDNA mixture is prepared and added to the cells on the same day as changing the media.

7.2. Duration

45 min active time; 4 days total

1.2.1 Dilute 9 µg of PEI 'Max' into a total volume of 150 µl of Opti-MEM. The amount of PEI can be varied.
1.2.2 Dilute 3 µg of DNA into a total volume of 150 µl of Opti-MEM.
1.2.3 Add the diluted PEI 'Max' to the diluted DNA.
1.2.4 Incubate the mixture at room temperature for 30 min.
1.2.5 Carefully add the PEI-DNA mixture to a well of adherent cells. Take care to gently pipette the solution down the side of the well and not on top of the cells, so as not to disrupt the adherent cells.
1.2.6 Return the dish to the 5% CO_2 incubator.

7.3. Tip

Opti-MEM can be replaced with Hybridoma Media without serum.

7.4. Tip

The protocol outlined here uses a 3:1 ratio of PEI to DNA (w/w). We have found this ratio to be optimal for most genes we have expressed. However, this ratio should be screened for each gene tested. We routinely screen ratios between 1:1 and 5:1.

7.5. Tip

In general, use 1 µg of DNA per 1 ml of culture to be transfected. PEI and DNA should each be diluted into 1/20 of the total culture volume before being combined.

7.6. Tip

Small-scale transfections can be performed with suspension-adapted cells. The protocol for small scale is essentially the same. For suspension culture, we use square plastic bottles designed to hold 125 ml; however, we add only 5–12 ml of cell medium for optimal aeration and agitation.

See Fig. 18.3 for the flowchart of Step 1.2.

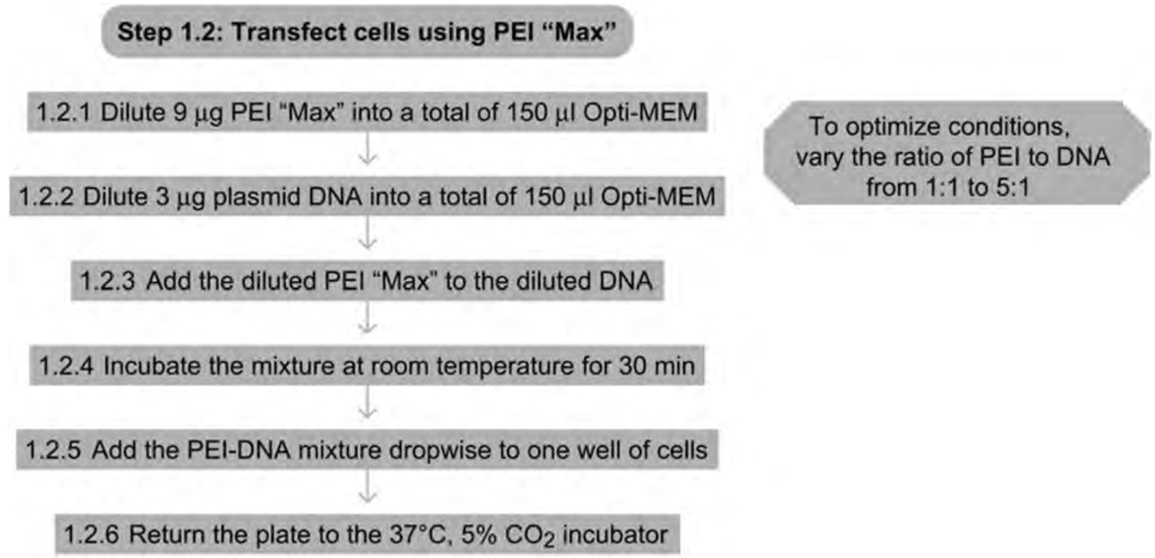

Figure 18.3

Flowchart of Step 1.2.

8. STEP 1.3 HARVEST CELLS AND ANALYZE PROTEIN EXPRESSION

8.1. Overview

Harvest and lyse cells (see Lysis of mammalian and Sf9 cells). Analyze protein expression by Western blotting (see Western Blotting using Chemiluminescent Substrates).

8.2. Duration

1–2 days

1.3.1 96 h after transfection, collect samples to be analyzed. For secreted proteins collect and save the media. For membrane or intracellular proteins, remove media. Wash the cells with PBS and lyse the cells.
1.3.2 Analyze protein expression by Western blotting or ELISA as appropriate.

8.3. Tip

HEK293 GnTI-cells can be resuspended by pipetting gently up and down with a serological pipette. CHO-S cells adhere more tightly to the dish and need to be manually resuspended with a cell scraper.

8.4. Tip

Some protocols call for trypsin digestion to detach the *cells from the dish. This can be avoided by manual scraping of the cells. Trypsin could degrade the expressed protein if it is a membrane protein.*

8.5. Tip

Once a cell type, media, and optimal ratio of PEI to DNA are established, this protocol can be repeated and samples taken between 24 and 96 h posttransfection to optimize the length of expression.

See Fig. 18.4 for the flowchart of Step 1.3.

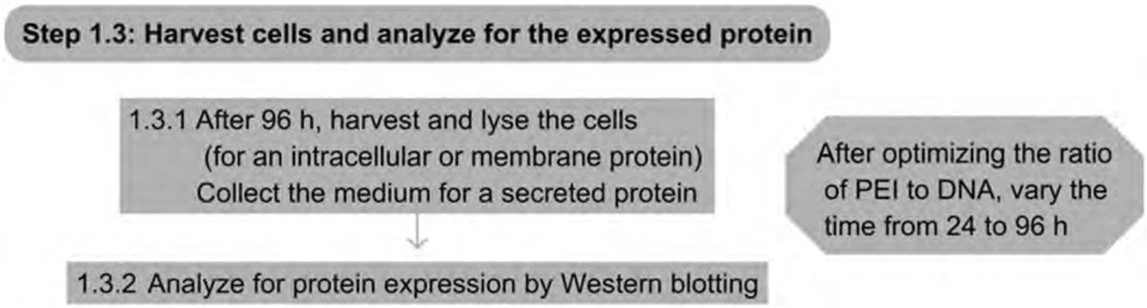

Figure 18.4

Flowchart of Step 1.3.

9. STEP 2 LARGE-SCALE TRANSIENT TRANSFECTION OF SUSPENSION CELLS

9.1. Overview

Preparative scale expression of protein in suspension culture. For this protocol, the parameters optimized in Step 1 are expanded to larger volume cultures. You will need 400 ml of cells at a density of 2–3 × 10^6 cells ml^{-1}.

9.2. Duration

4–8 days

10. STEP 2.1 PREPARE THE CELLS TO BE TRANSFECTED

10.1. Overview

Harvest and count suspension cells to ensure that they are at the proper density. Centrifuge the cells and resuspend them in a total of 360 ml of fresh suspension growth medium.

10.2. Duration

30 min

2.1.1 Grow 400 ml of cells in the appropriate suspension growth medium to a density between 2 and 3 × 10^6 cells ml^{-1}.
2.1.2 Transfer the cell suspension to sterile centrifuge bottles.
2.1.3 Spin the cells at 200 × g at room temperature for 5 min. Aspirate the supernatant.
2.1.4 Add 25 ml of the appropriate fresh suspension growth medium.
2.1.5 Gently resuspend the cells with a serological pipette.
2.1.6 Add the cells to 335 ml of fresh suspension growth medium in a sterile square bottle.
2.1.7 Do not tighten the bottle cap all the way.
2.1.8 Place the cells into a 37 °C incubator shaker set at 8% CO_2, with shaking at 130 rpm.

See Fig. 18.5 for the flowchart of Step 2.1.

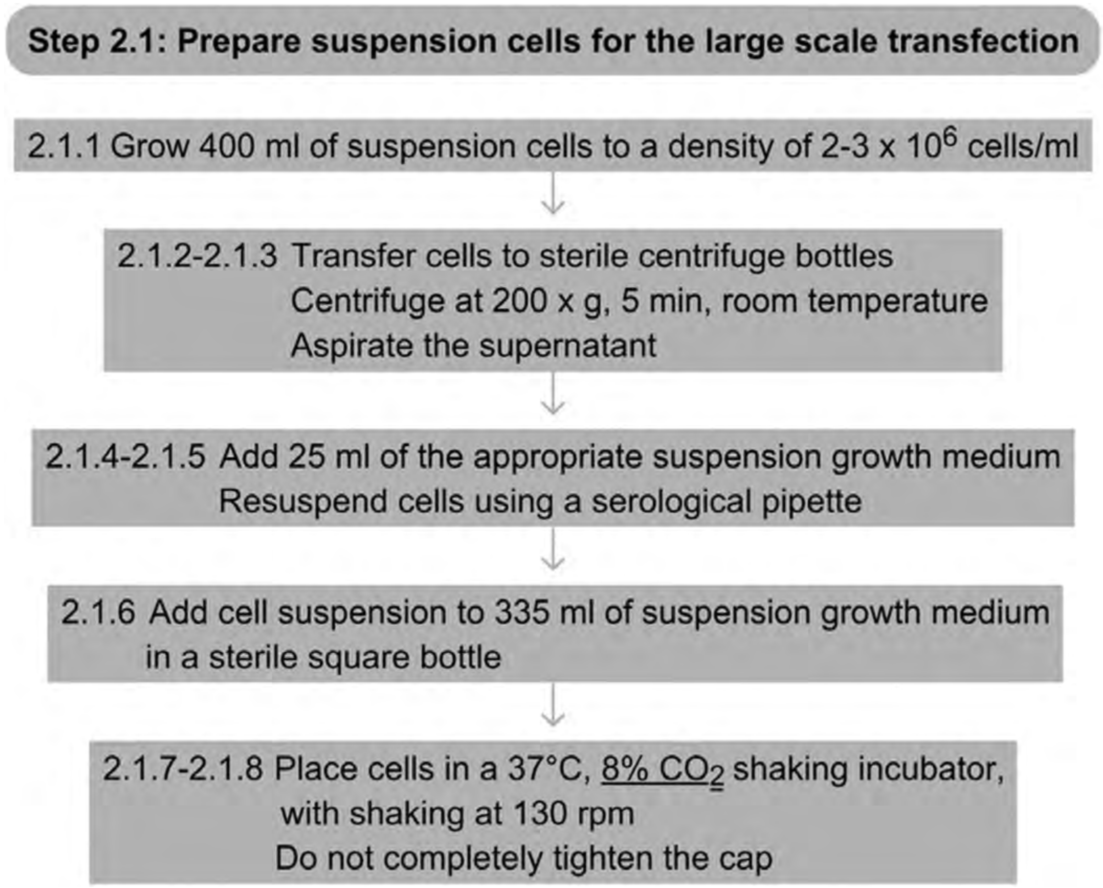

Figure 18.5

Flowchart of Step 2.1.

11. STEP 2.2 TRANSFECT CELLS

11.1. Overview

Transfect the cells using the optimal ratio or PEI to DNA as determined above.

11.2. Duration

45 min active time, expression time as determined above

2.2.1 Dilute 400 µg of DNA in a total volume of 20 ml of Hybridoma SFM (without serum).
2.2.2 Dilute the appropriate amount of PEI 'Max,' as determined in Step 1, into a total volume of 20 ml of Hybridoma SFM (without serum).
2.2.3 Add the diluted PEI 'Max' to the diluted DNA and mix.
2.2.4 Incubate the mixture at room temperature for 30 min.
2.2.5 Add the PEI-DNA mixture to the suspension cells from Step 2.1.8.
2.2.6 Return the cells to the 37 °C incubator shaker, shaking at 130 rpm.

See Fig. 18.6 for the flowchart of Step 2.2.

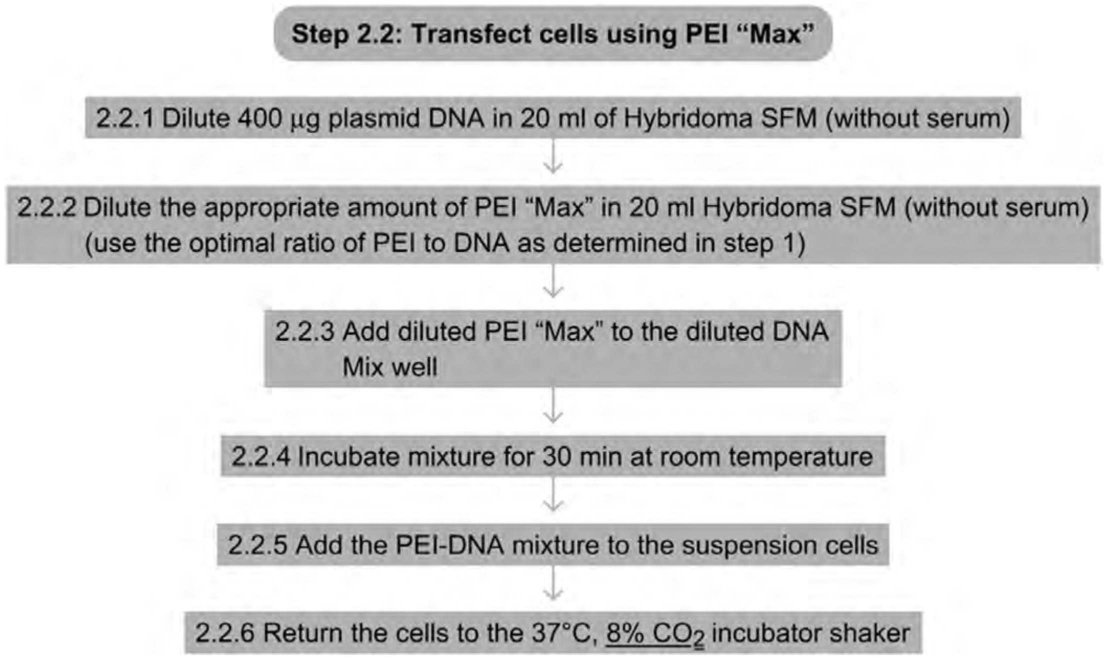

Figure 18.6

Flowchart of Step 2.2.

12. STEP 2.3 HARVEST CELLS AND PROCESS PROTEIN AS NEEDED

12.1. Overview

Harvest the cells (or medium for a secreted protein). Purify the protein or process as needed for downstream applications (see Salting out of proteins using ammonium sulfate precipitation, Using ion exchange chromatography to purify a recombinantly expressed protein, Gel filtration chromatography (Size exclusion chromatography) of proteins, Use and Application of Hydrophobic Interaction Chromatography for Protein Purification or Hydroxyapatite Chromatography: Purification Strategies for Recombinant Proteins, or look up the chapters on affinity purification if tags have been added to the protein: Purification of His-tagged proteins, Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein (MBP) tag, Purification of GST-tagged proteins, Protein Affinity Purification using Intein/Chitin Binding Protein Tags, Immunoaffinity purification of proteins or Strep-tagged protein purification).

12.2. Duration

About 1 h

2.3.1 After the appropriate amount of time, as determined in Step 1, centrifuge the cells at 200 ×g for 5 min at room temperature.
2.3.2 If the protein is secreted, collect the medium. Centrifuge the cells at 200 × g for 5 min at room temperature and sterile-filter the medium through a 0.22-µm filter. Add sodium azide to 0.02%. The medium can be stored at 4 °C for months until needed.
2.3.3 If the protein is to be purified, the cell pellet should be flash-frozen in liquid nitrogen and stored at −80 °C until needed.

12.3. Tip

In suspension cultures, single cells are transfected more efficiently than cells that have clumped together during growth. Growth conditions may need to be optimized for single cell growth.

12.4. Tip

Square bottles should be autoclaved in two consecutive dry cycles (45 min each, dry 15 min each) with the lids as loose as possible without falling off. They should be allowed to cool completely in the laminar flow hood before tightening the lids. If the bottles collapse inward, cells will not grow well.

12.5. Tip

To generate more protein, we have found that 24 h posttransfection the cells can be diluted between 1:2 and 1:5 in the appropriate media. The effect of dilution and optimal dilution ratios should be determined empirically.

See Fig. 18.7 for the flowchart of Step 2.3.

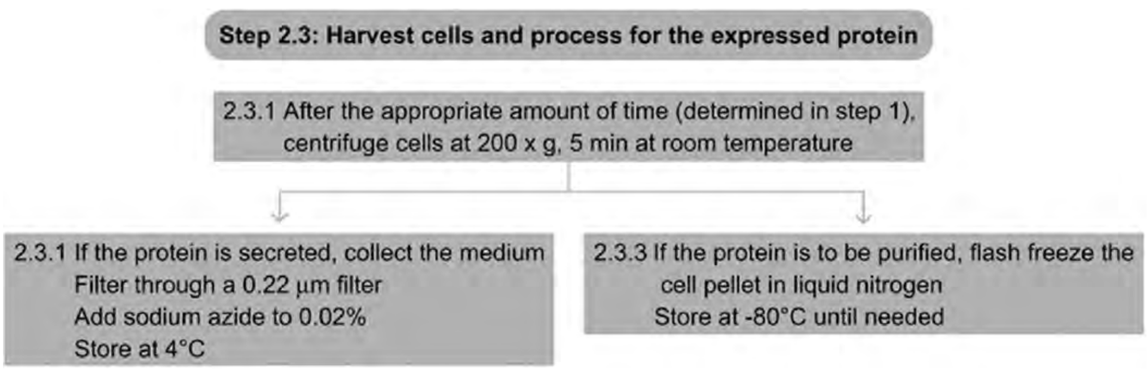

Figure 18.7

Flowchart of Step 2.3.

Referenced Protocols in Methods Navigator

Small-scale Expression of Proteins in *E. coli*

Rapid creation of stable mammalian cell lines for regulated expression of proteins using the Gateway® Recombination Cloning Technology and Flp-In T-REx® lines

Generating mammalian stable cell lines by electroporation

Molecular Cloning

Transformation of Chemically Competent *E. coli*

Transformation of *E. coli* via electroporation

Isolation of plasmid DNA from bacteria

Explanatory Chapter: Nucleic Acid Concentration Determination

Lysis of mammalian and Sf9 cells

Western Blotting using Chemiluminescent Substrates

Salting out of proteins using ammonium sulfate precipitation

Using ion exchange chromatography to purify a recombinantly expressed protein

Gel filtration chromatography (Size exclusion chromatography) of proteins

Use and Application of Hydrophobic Interaction Chromatography for Protein Purification

Hydroxyapatite Chromatography: Purification Strategies for Recombinant Proteins

Purification of His-tagged proteins

Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein (MBP) tag

Purification of GST-tagged proteins

Protein Affinity Purification using Intein/Chitin Binding Protein Tags

Immunoaffinity purification of proteins

Strep-tagged protein purification

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Exhibit O

Characterization of L1-Ribonucleoprotein Particles

Article *in* Methods in molecular biology (Clifton, N.J.) · February 2016

DOI: 10.1007/978-1-4939-3372-3_20

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Chapter 20

Characterization of L1-Ribonucleoprotein Particles

Martin S. Taylor, John LaCava, Lixin Dai, Paolo Mita, Kathleen H. Burns, Michael P. Rout, and Jef D. Boeke

Abstract

The LINE-1 retrotransposon (L1) encodes two proteins, ORF1p and ORF2p, which bind to the L1 RNA in *cis*, forming a ribonucleoprotein (RNP) complex that is critical for retrotransposition. Interactions with both permissive and repressive host factors pervade every step of the L1 life cycle. Until recently, limitations in detection and production precluded in-depth characterization of L1 RNPs. Inducible expression and recombinant engineering of epitope tags have made detection of both L1 ORFs routine. Here, we describe large-scale production of L1-expressing HEK-293T cells in suspension cell culture, cryomilling and affinity capture of L1 RNP complexes, sample preparation for analysis by mass spectrometry, and assay using the L1 element amplification protocol (LEAP) and qRT-PCR.

Key words LINE-1, Ribonucleoprotein, Affinity purification, Protein complexes, Interactomics, Cryomilling, Mass spectrometry, Metabolic labeling

1 Introduction

LINE-1 is the only active protein-coding transposon in humans. Hundreds of thousands of copies of L1 make up ~20 % of the human genome and are a source of genetic structural variation and between humans and instability in cancer [1–4]. Although most L1s are truncated or mutated, ~90 copies per cell are capable of replication [5]. As a streamlined DNA parasite with which we have coevolved since early eukaryotic existence [1], L1 encodes only two proteins and coopts cellular machinery in order to replicate. To defend against L1-mediated mutagenesis, our cells possess multiple mechanisms to suppress its activity [6–18]. Although teleological roles of retrotransposition in evolution are somewhat controversial [19–21], critical interactions with the host functions are highly conserved; for example, the PIP box, a motif required for L1 ORF2p-PCNA binding and retrotransposition, is conserved from corn to humans [6].

Jose L. Garcia-Pérez (ed.), *Transposons and Retrotransposons: Methods and Protocols*, Methods in Molecular Biology, vol. 1400, DOI 10.1007/978-1-4939-3372-3_20,

L1 ORFs bind the L1 mRNA to form a ribonucleoprotein (RNP) complex [22, 23]. ORF1p is a trimeric nucleic acid binding protein that is highly expressed in human cancers and cell culture models and thought to have chaperone activity; it is required for retrotransposition but its precise role is unknown [24–29]. ORF2p possesses both endonuclease and reverse transcriptase activities [30, 31] and, in contrast to ORF1p, is expressed at such low levels even after overexpression (due to an unconventional translation mechanism) that detection was a technical barrier in the field until recently [6, 32, 33]. However, the combination of a tetracycline-inducible promoter and improved epitope tagging now makes detection and purification of both ORFs routine [6], reviewed in [34]. A synthetic codon-optimized L1, *ORFeus*-Hs [35, 36], produces ~40-fold more L1 RNA and ORF2p and was critical to establishing protocols for purification of ORF2p; interactors were similar to native L1RP [6].

Here, we outline our methods for suspension production, cryomilling, and affinity capture of L1 RNPs with subsequent characterization by mass spectrometry, the L1 element amplification protocol (LEAP) [23], and quantitative real-time reverse transcription PCR (qRT-PCR). Suspension production improves cell densities as compared to adherent cell culture, and allows for sufficient cell material for solid-phase lysis under liquid N_2 by cryomilling. This approach has a number of practical advantages over liquid-phase lysis including reduced background in coimmunoprecipitation and the ability to store the milled cell powder at −80 °C, allowing repeated experiments to be performed from the same sample without the need to produce more starting material [37, 38].

We express inducible L1 in Tet-On HEK-293_{LD} cells using pCEP4-based episomal vectors and either large-scale transient transfection or quasi-stable episomal puromycin-selected cell pools [6]. Because no Tet-On HEK-293T cells were available commercially, we produced this line using a linearized pTet-On Advanced (Clontech), modified to contain blasticidin resistance instead of neomycin (pLD215). Cells are maintained in square bottles on an orbital shaker [39]. Alternative systems include GNTI-HEK293S cells, HEK-293F cells (Life Technologies), and the T-REx system (Life Technologies), and can be achieved in spinner flasks, conical flasks, and wave bags [40, 41].

For L1 affinity isolation, we couple antibody to functionalized micron-scale paramagnetic beads with relatively inert surfaces (Dynabeads, Life Technologies). After coupling, antibody is immobilized on the surface of the bead. For the isolation of protein complexes, these beads provide a number of advantages over agarose and porous synthetic resins (which both contain antibody bound within the pores) including the ability to bind and release larger complexes, faster binding and release, and reduced background [37, 38, 42]. Switching to this medium was critical for the successful characterization of L1 RNPs.

L1 RNPs purified by affinity capture provide the purest, most active L1 elements reported to date and are excellent starting material for assay by mass spectrometry, LEAP, and qRT-PCR [6]. We provide several of our working protocols for L1 sample preparation and analysis.

2 Materials

2.1 Suspension Cell Culture

1. Humidified CO_2-controlled tissue culture incubator.
2. Orbital shaker platform at 130 rpm fitted with racks.
3. 20 mm 40-place test tube racks.
4. Diagonal cutting pliers, flat wood file, bandsaw (for modifying racks).
5. Corning Pyrex 1 L glass bottles.
6. 7×® Cleaning solution (Bellco Glass).
7. Hybridoma SFM medium (Life Technologies).
8. Freestyle 293 Medium (Life Technologies).
9. Opti-MEM Reduced Serum Medium.
10. TrypLE Express (Life Technologies).
11. Certified tetracycline-Free FBS (Tet-free FBS).
12. DMEM medium.
13. Phosphate-buffered saline (PBS).
14. PEI Max (MW 40,000), Polysciences: Two grams is enough to transfect >600 L.
    - (a) To prepare working 1 mg/mL PEI Max solution:
    - (b) Dissolve 100 mg PEI Max in 90 mL ddH_2O.
    - (c) Adjust pH to 7.0 using 1 M NaOH.
    - (d) Adjust volume to 100 mL, filter sterilize, and store at 4 °C.
    - (e) *NEVER FREEZE PEI working stock*. Working stocks can be used for up to 6 months if stored at 4 °C.

2.2 Cell Harvest

15. Large-volume floor centrifuge with appropriate rotor (e.g., 4 × 1 L, 6 × 500 mL).
16. 16ga needles.
17. Luer-lock syringes, 5 mL, 10 mL, or 30 mL.
18. Luer-lock syringe end caps (BioRad).
19. Liquid nitrogen and Dewar flask.
20. Gloves for handling liquid nitrogen.
21. Small Styrofoam box.

2.3 Cryomilling

22. Ice pan (Fisher).
23. RETSCH Planetary ball mill PM 100 or PM 100 CM.
24. RETSCH Stainless steel grinding balls 20 mm diameter.
25. RETSCH Stainless steel "comfort" grinding jars 50 mL and/or 125 mL.
26. Stainless steel measuring spoons for small amounts (e.g., "hint, pinch, dash").
27. Stainless steel spatulas.
28. Extra-large forceps.

2.4 Coupling of Magnetic Medium (Dynabeads)

29. Dynabeads M270 Epoxy (Life Technologies).
30. Anti-Flag M2 Antibody (Sigma). *See* **Note 1.**
31. Anti-ORF1 Antibody 4H1 (obtained from Kathleen H. Burns).
32. Magnetic separator for microcentrifuge tubes (Dynamag 2, Life Technologies).
33. Magnetic separator for 15 mL conical tubes (Dynamag 15, Life Technologies). *See* **Note 2**.
34. Zeba Spin Desalting Columns 7 K MWCO (Thermo) or chromatography system with preparatory-scale desalting column.
35. Nutating mixer or orbital shaker.
36. Rotating test tube wheel in a 37 °C environment.
37. 100 mM Sodium phosphate buffer pH 7.4 (makes 1 L):
    (a) 2.62 g Sodium phosphate monobasic monohydrate.
    (b) 14.42 g Sodium phosphate dibasic dihydrate.
    (c) Dissolve in 900 mL ddH_2O, adjust pH if necessary with HCl and NaOH, and adjust to 1 L.
38. 3 M Ammonium sulfate (in phosphate buffer) (makes 100 mL):
    (a) 39.6 g Ammonium sulfate.
    (b) Dissolve in 0.1 M sodium phosphate buffer (pH 7.4) and adjust to 100 mL.
39. 10× PBS—pH 7.4. *See* **Note 3** (makes 1 L):
    (a) 2.62 g Sodium phosphate monobasic monohydrate.
    (b) 14.42 g Sodium phosphate dibasic dihydrate.
    (c) 87.8 g Sodium chloride.
    (d) Dissolve in 900 mL with ddH_2O, adjust pH if necessary with HCl and NaOH, and adjust to 1 L.
40. Resuspension buffer (PBS, 50 % glycerol, 0.5 mg/mL BSA; makes 10 mL):
    (a) 1.0 mL 10× PBS.
    (b) 6.3 g glycerol (place tube + rack on a balance, tare, and pipet).

(c) 5 mg BSA.
(d) ddH_2O to 10 mL.

41. PBS + 0.5 % Triton X-100 (w/v) in 100 mL.
42. 100 mM Glycine-HCl, pH 2.5 (adjust pH with HCl).
43. Triethylamine.
44. 10 mM Tris–HCl, pH 8.8.
45. 10 % (w/v) Sodium azide (NaN_3).

2.5 Affinity Capture (See Note 4)

46. HEPES buffer, pH 7.4.
47. Sodium chloride.
48. Triton X-100.
49. Protease inhibitor cocktail: Complete EDTA-free (Roche).
50. Ultrasonic liquid processor with micro tip (Branson Sonifier or similar).

2.6 LEAP (L1 Element Amplification Protocol) and Real-Time Reverse Transcription PCR (qRT-PCR)

51. LEAP reaction mixture (Table 1), shown for ten reactions.
52. Primers for LEAP and qRT-PCR (*see* Table 2).
53. SuperScript III Reverse Transcriptase (Life Technologies) or similar.
54. FastStart Taq DNA Polymerase (Roche Applied Science) or similar.
55. TRIzol Reagent (Life Technologies).
56. RNA from GFP-transfected human cells, purified (*see* **Note 5**).

Table 1
LEAP reaction mixture for ten reactions

Sample	Final concentration	Stock concentration	µL each	×10
RNP prep			2	
Tris pH 7.5	50 mM	1 M	2.5	25
KCl	50 mM	1 M	2.5	25
$MgCl_2$	5 mM	1 M	0.25	2.5
DTT	10 mM	1 M	0.5	5
3′ Anchor primer	0.4 µM	10 µM	2	20
RNAsin	20 U/rxn	40 U/µL	0.5	5
dNTPs	0.2 mM	10 µM	0.5	5
Tween 20	0.05 %	10 %	0.25	2.5
ddH_2O			39	390

Table 2
Primers for LEAP and qRT-PCR

Name	Sequence
JB11560	5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTT-3′
JB11564	5′-GCGAGCACAGAATTAATACGACTC-3′
JB14067	5′-GGATCCAGACATGATAAGATACATTGATGA-3′
JB13415	5′-GCTGGATGGAGAACGACTTC-3′
JB13416	5′-TTCAGCTCCATCAGCTCCTT-3′
JB13417	5′-CTGATCAGCCGCATCTACAA-3′
JB13418	5′-TGGTCTTGATCTGCATCTCG-3′
JB13766	5′-ACGTAAACGGCCACAAGTTC-3′
JB13767	5′-AAGTCGTGCTGCTTCATGTG-3′

57. StepOne Plus Instrument or similar (Life Technologies) and appropriate reaction plates.
58. Fast SYBR Green Master Mix (Life Technologies) or similar.
59. RNAseZap (Life Technologies) or similar.

3 Methods

3.1 Suspension Culture of HEK-293T Cells

Suspension culture allows production of large amounts of human cell pellets with minimal work, waste, and cost when compared to monolayer adherent growth. We use a square bottle system in an orbital shaker, modified from [39] (Fig. 1a). For comparison, one confluent 15 cm plastic adherent culture dish provides about 100 mg wet cell weight (cell pellet, WCW), corresponding to 20–30 million cells, and uses 20 mL of media. To produce 1.5 g of cell pellet, 15 disposable dishes and 300 mL of media are required, and scale up, induction, and harvest are laborious. In suspension, cell densities of 4–5 million cells/mL are readily achieved in log phase using reusable 1 L glass bottles, yielding 4–5 g WCW from 333 mL media. Thus, each bottle is the equivalent of approximately 50 culture plates. The total yield from 48 culture bottles (Fig. 1a) was approximately 250 g WCW and was harvested in 90 min by one person; an entire incubator full of plates (Fig. 1b) produced 16 g WCW and was harvested in 4 h by two people.

We culture Tet-On HEK-293T$_{LD}$ cells in Freestyle 293 medium supplemented with 1 % tetracycline-free FBS and 2 mM l-glutamine (suspension medium, *see* **Note 6**). Basic shaker setup and maintenance are described below. Initially, for each construct we

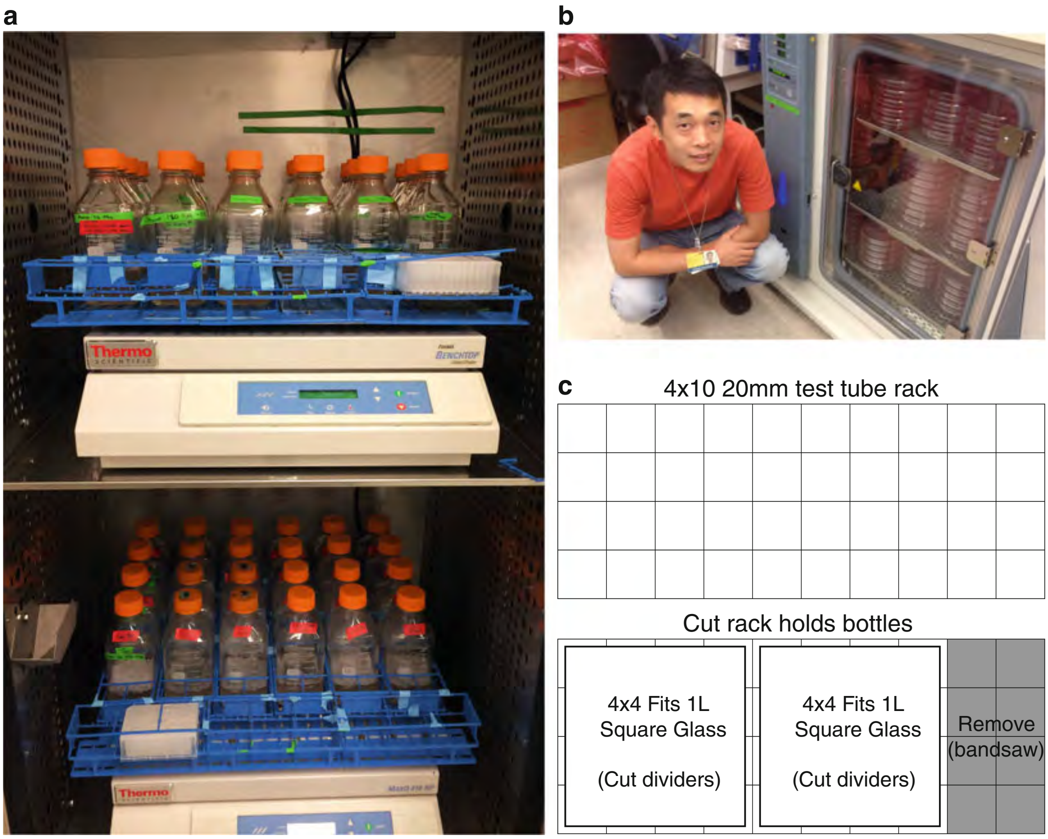

Fig. 1 Suspension culture of HEK-293T$_{LD}$ cells using square glass bottles. (**a**) Orbital shaker bottle setup in a CO_2-controlled humidified incubator cabinet. Total yield from 48 bottles was approximately 250 g wet cell weight (WCW), the equivalent of 2500 15 cm adherent culture plates. (**b**) Dr. Lixin Dai proudly showcases an incubator full of adherent culture plates for harvest. Harvesting this entire incubator full of cells yielded 16 g WCW, which can now be accomplished in three to four suspension bottles. (**c**) Diagram of modifications to Nalgene 4 × 10 configuration 20 mm test tube racks to fit square glass bottles. Dividers are cut with diagonal cutting pliers and smoothed with a file. Sections of rack can be removed with a bandsaw. The diversity of available rack sizes and shapes allows adaptation of this strategy for many size bottles

transfected, puromycin-selected, and scaled up adherent cultures to suspension. This protocol is effective but time consuming. Subsequently, we found that large-scale transient transfection using PEI-MAX (Polysciences) provides equivalent per-cell yields with much less work. Both protocols are described in separate sections below (*see* **Note** 7).

3.1.1 Square Bottle Orbital Shaker Setup

We use a Thermo Forma Model 416 orbital shaker in a 5 % CO_2 humidified incubator cabinet, shaking at 130 rpm. After repeated inconsistency issues with failed transfections and poor growth in plastic bottles made of polypropylene, polyethylene, and other plastics, we can only recommend culture in square Corning Pyrex

glass bottles. Bottles are held in place using autoclavable test tube racks, cut to size. The racks last for a number of years in regular use before needing to be replaced.

1. Cut tube racks to fit bottle configurations (Fig. 1c). Use diagonal cutting pliers to remove undesired dividers and then a flat wood file to smooth sharp edges. To shorten racks, cut to length with a bandsaw. We start with 10 × 4 racks and cut most racks to fit two 1 L glass bottles, a configuration with two 4 × 4 openings as shown. Other configurations or other size starting racks would allow use of alternate or smaller bottles.
2. Clean and autoclave racks before installation.
3. Install racks to shaker platform using stainless steel flat-head machine screws and, if needed, washers. The bottom of the racks may need to be drilled.
4. Use paper tape between the racks to hold weak sections together.

3.1.2 Bottles and Volumes

1. Clean freshly gloved hands with 70 % ethanol before going into the shaker cabinet.
2. Media is maintained at up to 33 % of the indicated capacity for optimal mixing and gas exchange.
   (a) Minimum volume is 5 % of indicated capacity (50 mL for 1 L bottles).
   (b) Cell shearing is inversely proportional with bottle size; too little volume can result in excessive shearing.
3. Bottle caps are kept loose to allow gas exchange, but not loose enough to easily fall off.
4. The outsides of bottles are wiped clean before returning to the shaker.
5. After use, bottles must be immediately rinsed and filled with tap water. Bottles are left soaking until they are ready to clean. A small number of "beached" cells stuck to the side of the glass at the media-air interface is normal for bottles that have been used for a number of weeks.
6. When a batch of bottles is ready for sterilization, bottles are thoroughly scrubbed with a brush cleaned using 1 % solution of 7× cleaner, rinsed six times using tap water, and finally rinsed with deionized water.
7. Bottles are sterilized by autoclaving using a dry cycle at 121 °C, with 45-min sterilization and 15-min drying. *See* **Note 8**.
8. In the event of contamination, bottles are soaked in 10 % bleach for at least 2 h and then washed and autoclaved as above. *See* **Note 9**.

3.1.3 General Cell Line Maintenance

1. Most operations are done by pouring. Be careful to consider that only the threads of the bottle are sterile.
2. Do not allow non-sterile bottle sides to be positioned over the mouth of an open bottle. If a bottle is to be totally emptied, we pipet the last ~40 mL.
3. Caps are stored face-down on the hood. Remember that the hood surface and the rim of the cap are never sterile but the cap threads and inside are sterile.
4. This media has no pH indicator dye, but one can be added if desired.
5. Cells are generally maintained at a density of ~0.2–4 million/mL (*see* **Note 10**). Cells will grow to ~7 million/mL [39] but growth slows after ~5 million/mL.
6. We do not spin the cells down to fully exchange media. Passaging is done by dilution. Centrifugation is time consuming, risks contamination, and is generally not helpful.
7. A typical 1:5 split of a 333 mL culture is done as follows:
   (a) Count the cells.
   (b) Fill to 1 L (there is an indentation in the bottles at exactly this volume).
   (c) Cap tightly and gently shake to mix.
   (d) Pour 200 mL of diluted culture into each bottle.
   (e) Fill each bottle to 333 mL.
8. Antibiotics and/or antimycotics can only be used if cells are not to be transfected in suspension because the combination of PEI and Pen-strep is toxic. Most of our cultures are antibiotic free.

3.1.4 Counting Suspension HEK-293Ts with a Hemocytometer

HEK-293Ts grow in small clumps of 1–30 cells in suspension. Accurate counting requires dissociation of the clumps by gentle trituration using a pipet. Due to differences in light scattering by different clump sizes, optical density is not an accurate measure of cell number. We visualize the cells before and after dissociation because shearing in the dissociation protocol lyses a small fraction of the cells.

1. Using a 1 mL serological pipet, aliquot 200 μL (*see* **Note 11**) of culture to a clean microcentrifuge tube (*see* **Note 12**).
2. Mix by flicking, and pipet 10 μL onto one side of the hemocytometer.
3. With a 200 μL pipet, set the volume to ~150 μL and triturate 30 times to break up clumps. Try not to foam.
4. Pipet 10 μL onto the remaining half of the hemocytometer.

3.1.5 Suspension Transient Transfection Using PEI Max

Transfection is done using 1 μg DNA/mL media. Transfection complexes are prepared in 1/20th the culture volume hybridoma SFM with a 3:1 ratio of PEI Max to DNA (*see* **Note 13**). A transfection protocol for 1 L culture (three 1 L bottles, each containing 333 mL culture) is outlined below.

1. Prep DNA. High-quality endotoxin-free DNA is critical to success. We have had best results with PureLink HiPure Maxi and Giga prep kits (Life Technologies) using the manufacturer's protocol (*see* **Note 14**).
2. Day 1:
   (a) Grow cells to 2.5–3.5 million/mL. Count the cells.
   (b) Warm 50 mL of hybridoma SFM to room temperature (*see* **Note 15**).
   (c) Dilute 50 μg DNA in the hybridoma media. Mix well (*see* **Note 16**).
   (d) Add 150 μL 1 mg/mL PEI Max (pH 7.0). Mix well.
   (e) Incubate for 15 min at room temperature to allow DNA-PEI complex to form.
   (f) Pipet 16.7 mL into each 333 mL culture and return to the incubator.
3. Days 2–3 or 2–5:
   (a) Option 1: Induce cells by adding 1 μg/mL doxycycline. Harvest 24 h later on day 3 (*see* **Note 17**).
   (b) Option 2: Split cells 1:3 on day 2, induce on day 4, and harvest 24 h later on day 5 (*see* **Note 18**).

3.2 Adherent Transfection, Selection, and Adaptation to Suspension

Transfection is done on 6-well plates and followed by a puromycin selection done simultaneously with scale-up and transition to suspension medium. Adherent cells are maintained in DMEM supplemented with 10 % tetracycline-free FBS and penicillin-streptomycin (adherent medium).

1. Transfection (*see* **Note 19**):
   (a) Day 1: Plate 300,000 cells per well 6-well plates. Plate four wells per construct. Plate an additional four wells for a killing control.
   (b) Day 2: Prepare transfection mixtures of 400 μL Opti-Mem, 4 μg of DNA, and 12 μL Fugene HD (3:1 reagent:DNA ratio), following the manufacturer's protocol. After complex formation, add 100 μL of transfection mixture to each well and mix by rocking the plate.
   (c) Day 3:
      i. Dissociate the cells by banging or by using TrypLE Express (*see* **Note 20**).

ii. Pool the four wells used for each construct. Plate on 10 cm dishes or T-75 culture flasks in a 50:50 mixture of suspension medium (*see* **Note 21**) and adherent medium, supplemented with 1 μg/mL puromycin; higher concentrations may also be used [43] (*see* **Note 22**). For a good transfection, >80 % of cells will survive this harsh transition.

(d) Day 5–6: Control (untransfected) cells should be almost completely dead, with few cells adhered. Once transfected cells are >80 % confluent, split onto 2 × 15 cm dishes using an 80:20 mixture of suspension medium and adherent medium, supplemented with puromycin (*see* **Notes 23** and **24**).

(e) Days 7–9: Once the cells are confluent, adapt to suspension:

i. Prepare 50 mL of a 90:10 mixture of suspension medium and adherent medium (*see* **Note 25**).

ii. Dissociate and count the cells. Remove the media.

iii. Resuspend the cells in 10 mL media and triturate aggressively to break up clumps. Increase volume to 50 mL (typically ~1 million/mL), transfer to a 1 L bottle, and immediately put in the shaker.

(f) Days 9–11: Check the cells after 2 days in suspension. Typical density should be >2 million per mL. Cultures are typically very clumpy at this point, but healthy and doubling every 24–36 h. Sometimes the clumps are large enough to see by the naked eye. The goal of the following steps is to reduce the serum concentration and select nonclumping cells.

i. Transfer 25 mL of the culture (50 mL) into each of the two 50 mL conical tubes.

ii. Triturate five times.

iii. If the cells are very clumpy, vortex three pulses of 5 s (maximum intensity) to break up clumps.

iv. Transfer tubes to a rack in the hood. Count the cells. While counting, allow the cells to settle for a total of 5 min; large clumps will settle rapidly.

v. Split the cells to 0.5 million per mL, adding only suspension medium.

vi. Remaining cells may be used for a test induction at this point.

(g) Expand cells to desired volume and induce at 3.5–4.5 million/mL with 1 μg/mL doxycycline. Harvest 24 h later (*see* **Note 26**).

3.3 Harvest of Suspension Cells to Make Liquid Nitrogen "BB's" (See Note 27)

Cells are spun down in syringes and injected into tubes containing liquid nitrogen [38]. A video protocol of this process done in yeast cells is available at http://www.ncdir.org/public-resources/protocols/. The below protocol is modified for human cells.

1. Gather an ice bucket and clean centrifuge bottles.
2. Count cells. Counts are useful for normalization and blotting.
3. Transfer a 1 mL aliquot(s) of culture for western blotting to a microcentrifuge tube. Spin at $500 \times g$ for 30 s, aspirate the media, and store on ice until freezing is convenient.
4. Spin the cultures at $1000 \times g$ for 10 min at 4 °C to pellet. We use both 4×1 L and 6×500 mL centrifuge rotors (*see* **Note 28**). Pour off the media.
5. Resuspend the pellets in a minimal volume of PBS (approximately equal volume to the pellet). Pool resuspended cells.
6. Pellet cells inside syringes.
   (a) Select syringe(s) for cell pelleting. Five milliliter syringes are ideal for small samples. Use 10 mL or 30 mL syringes for large samples.
   (b) Remove the plungers and set aside.
   (c) Securely cap syringes with luer-lock end caps and place inside 50 mL conical tubes (*see* **Note 29**).
   (d) Spin at $200 \times g$ for 10 min at 4 °C. Spinning harder risks breaking the syringes. If cells are not well pelleted, spin again. Transfer syringes to an ice bucket.
   (e) Aspirate the PBS, leaving wet cells in the syringe (*see* **Note 30**).
7. Insert a conical tube rack in a small Styrofoam box. Fill with liquid N_2 (LN_2) to the top of the rack (*see* **Note 31**). Pre-label 50 mL conical tubes, transfer to the rack, and fill with LN_2 (*see* **Note 32**) (Fig. 2).
8. Pre-chill a small stainless steel spatula, standing it vertically in the liquid nitrogen.
9. Punch a number of holes in the caps of the tubes using a 16ga needle. This allows drainage of the LN_2 without loss of cells.
10. Inject the cells gradually into the tube containing LN_2 (Fig. 2) (*see* **Note 33**). If injected too fast, they will form large clumps. Use the pre-chilled spatula as needed to break apart any clumps.
11. Cap the tube using the punched lid. Careful! Pressure will build up and liquid can shoot out. Decant into a sink. Replace the punched lid with a new, intact lid and store tubes at –80 °C until cryomilling (*see* **Note 34**).

Fig. 2 Injection of pelleted human cells into liquid nitrogen to make "BB's." After centrifugation in a capped syringe, cells are injected into 50 mL conical tubes containing liquid nitrogen. Injection at a moderate rate prevents over-clumping of the cells. Use a pre-chilled spatula to break up any clumps

3.4 Cryomilling

We cryomill cells under liquid nitrogen in a Retsch PM 100 planetary ball mill; model PM 100 CM is also suitable. A newer model, the CryoMill, may be suitable for smaller samples. This protocol was initially developed for yeast, which are harder to break than mammalian cells due to the tough cell wall, and then adapted for mammalian cells with two stages of grinding [38]. Depending on the amount of cells to grind, we either use a 50 mL jar (~1–8 g cells) or a 125 mL jar (~5–30 g cells, Fig. 3a) (*see* **Note 35**). We present here a simplified protocol, after finding that the second stage of grinding was not necessary (Fig. 3b). Custom-made PTFE insulators (Fig. 3a) minimize warming of the sample during grinding and improve safety and performance (*see* **Note 36**). We use a homemade LN_2 decanter made using a spatula and 50 mL conical tube to pour LN_2 into and over the grinding jars [38].

1. Pre-clean grinding jar, lid, balls, two small steel spatulas, and large forceps using Windex glass cleaner or similar. Inspect the PTFE gasket for signs of damage. For a 50 mL chamber, use two 20 mm diameter balls. For a 125 mL chamber, use five 20 mm diameter balls.
2. Weigh the jar + insulators + balls and adjust PM 100 counterbalance accordingly.
3. Precool the jar, balls, spatulas, forceps, LN_2 decanter, and PTFE insulators in a clean Styrofoam box containing liquid LN_2 until the LN_2 stops boiling (*see* **Note 37**). Set up a working pan with LN_2 (*see* **Note 38**).

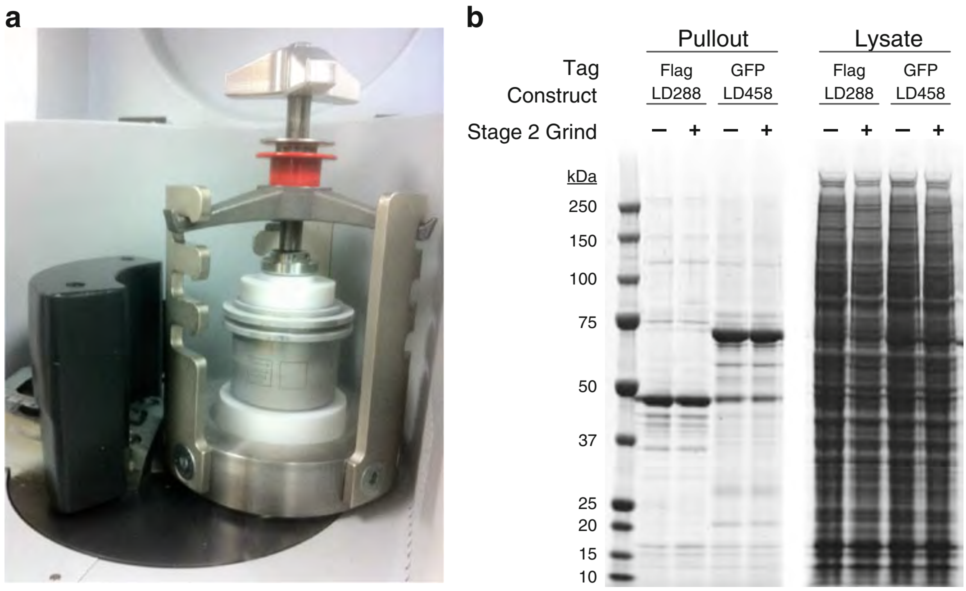

Fig. 3 Cryomilling setup for human cells. (**a**) Cryomilling apparatus in the Resch PM 100. 125 mL grinding jar is shown with custom PTFE insulators above and below. (**b**) Comparison of simplified one-stage and prior two-stage grinding protocols on protein extraction and affinity capture reveals equivalent results. HEK-$293T_{LD}$ cells expressing full-length L1 (ORFeus*HS* background) with ORF1p tagged with Flag (LD288) or GFP (LD258) were affinity captured (pullout) using respective epitope tag antibody-conjugated Dynabeads in our standard extraction solution and eluted under denaturing conditions. Total extracted lysate (lysate) before affinity capture is shown

4. Transfer the cold PTFE base to the grinder.
5. Transfer the frozen cell BB's into the grinding jar.
6. Fill with LN_2 to within ~0.5–1 cm of the top. Cover with the lid and Teflon top insulator, move jar plus lids *en bloc* onto the grinder, and clamp in place (*see* **Note 39**).
7. Pour LN_2 over the jar using the decanter.
8. Grind with three cycles of the following program: 400 rpm, 3 min, reverse rotation every 30 s, no interval breaks (*see* **Note 40**). Between grinding cycles, cool the jar as below:
   (a) Pour LN over the jar using the decanter to cool the lid and top.
   (b) Some pressure will have built up during the grinding. The jar may be gently hissing as pressure escapes: this is normal. Carefully remove the jar as in **step 9** below and transfer to the pan of LN_2 to recool.
   (c) Remove the lid and use a spatula to scrape any adhered powder back into the chamber. Do not scrape the gasket with a spatula: it will damage the PTFE seal. Submerse the lid to cool.

(d) Use a spatula to scrape around the lower corners of the jar, dislodging any packed cells.

(e) Refill the jar with LN_2 and reassemble as in **steps 6** and 7.

9. To remove the jar, slowly release clamping pressure (*see* **Note 41**). Transfer jar assembly to the pan of LN_2.
10. Put a pre-labeled 50 mL tube in a rack in the LN_2 pan. Remove steel balls with forceps, dislodging large chunks of grindate with a spatula.
11. Transfer grindate to conical tubes by pouring or with chilled spatulas or spoons. Once the sample is fully transferred, cap the tube loosely and move to a rack inside the Styrofoam box.
12. Store vertically at −80 °C overnight with the caps loose to allow LN_2 to evaporate, and then seal and store (*see* **Note 42**).

3.5 Conjugation of Dynabeads with Anti-Flag or Anti-ORF1 Antibody

This protocol was originally developed for bulk rabbit IgG [37] and has been adapted for use with precious/expensive antibodies, like anti-Flag M2. See also [44] for a general protocol. Nucleophilic side chains and N-termini on the antibody react with epoxide functional groups on the bead surface. It is critical that all other nucleophiles are absent from the buffer, or these will react with the beads and prevent antibody coupling. This includes tris, glycerol, azide, and other common antibody buffer components. It is safest to buffer exchange antibodies from commercial sources unless the absence of nucleophiles can be assured.

3.5.1 Antibody Buffer Exchange with Microcentrifuge Desalting Columns

We exchange the buffer twice to remove as much contaminant as possible. For large-scale couplings, we use a fast protein liquid chromatography (FPLC) system (ÄKTA, GE Healthcare) with a preparative-scale desalting column, but in the absence of this equipment, and for small, precious antibody samples, we use the below protocol. Alternative methods include ion exchange and dialysis.

1. Pre-equilibrate Zeba™ Spin Desalting columns in PBS three times according to the manufacturer's instructions.

   (a) Zeba™ columns have a maximum volume of 130 μL. Because we desalt twice, equilibrate two columns for every 130 μL. For example, for 250 μL antibody solution, equilibrate four columns.

2. Load, centrifuge, and recover exchanged antibody solution according to the manufacturer's instructions. Repeat once on fresh columns for a total of two exchanges (*see* **Note 43**).

3.5.2 Antibody Coupling

This protocol is for coupling of 300 mg Dynabeads. At the last step the beads are slurried by the addition of 2 mL buffer (*see* **Note 44**). We also routinely couple with 60 mg Dynabeads, slurried by the addition of 400 μL. For a 60 mg coupling, scale volumes in the

protocol linearly. Smaller volumes can also be used [44]. A commercial coupling kit for Dynabeads is available from Life Technologies; this uses proprietary components but is effective.

Day 1: Coupling

1. Calculate how much antibody to use. One milligram of Dynabeads M-270 Epoxy has been estimated to immobilize 7–8 μg of antibody during coupling (*see* **Note 45**). Coupling is not 100 % efficient and excess antibody appears to help drive the reaction [37]. For commercially available antibodies we use 10 μg antibody/mg Dynabeads (3 mg α-Flag for a 300 mg Dynabead coupling, *see* **Note 46**). We use 5 μg/mg for precious custom antibodies, and 15 μg/mg when we have the hybridoma.
2. Resuspend an entire bottle of 300 mg Dynabeads M270 Epoxy with 10 mL of 100 mM phosphate buffer, pH 7.4. Directly add buffer to the bottle and vortex bottle.
3. Transfer to a 15 mL falcon tube and wash the bottle twice with 2 mL phosphate buffer (*see* **Note 47**).
4. Shake bead suspension for 10 min on a nutating mixer or orbital shaker.
5. While the beads mix, prepare the *antibody mixture* (20 μL/mg Dynabeads, 6 mL total).
   (a) 2 mL 3 M Ammonium sulfate.
   (b) 3 mg antibody, double buffer-exchanged or supplied in phosphate buffer or PBS, free of glycerol and interfering species (see the manufacturer's instructions).
   (c) 0.1 M Phosphate buffer to 6 mL.
6. Transfer beads to a magnetic separator (*see* **Note 48**). Aspirate the buffer.
7. Wash again with 10 mL 100 mM phosphate buffer. Add buffer, vortex for 15 s, apply magnet, and aspirate; no incubation is necessary.
8. Add the *antibody mixture* to the beads. Seal and Parafilm the tube, and mix well.
9. Incubate overnight (18–24 h) on a rotating wheel at 37 °C (*see* **Note 49**).

Day 2: Bead Washing

1. Separate beads from the antibody mixture with a magnet. Carefully remove the antibody mixture and set aside in a clean tube: it still contains 30–50 % of the antibody, unreacted, which can be recovered for reuse.
2. Wash beads once with 12 mL 100 mM glycine pH 2.5. Add buffer, vortex briefly, and take it off as fast as possible.

3. Wash once with 12 mL 10 mM Tris–HCl, pH 8.8.
4. Prepare fresh 100 mM triethylamine: Add 168 μL stock to 11.8 mL ddH_2O. Apply, mix, remove, and proceed to the next step as fast as possible.
5. Wash the beads with 12 mL 1× PBS, incubating for 5 min on the nutator. Repeat a total of four times.
6. Wash twice with 12 mL PBS + 0.5 % Triton X-100, incubating each wash for 10 min on the nutator.
7. Resuspend beads in 2 mL *resuspension buffer:* PBS, 50 % glycerol, and 0.5 mg/mL BSA (*see* **Note 50**).
8. Mix well and aliquot 100 μL each into Eppendorf tubes. Store at −20 °C (*see* **Note 50**).

Reuse and Optional Antibody Recovery Using Protein G affinity (*see* **Note 51**). Approximately 30–50 % of antibody is unbound after coupling. We routinely save antibody mixtures for use in other assays such as immunoblotting and immunofluorescence. Alternatively, recovered antibody can easily be re-captured using Protein G affinity. Recovery also allows concentration of antibody and transfer into a more permanent storage buffer. We use Protein G Sepaharose Fast Flow (GE Healthcare) (*see* **Note 52**) following the manufacturer's instructions. The antibody sample should be diluted with an equal volume of PBS for binding. Based on 50 % antibody-Dynabead binding, ~125 μL resin is needed for recovery after a 300 mg Dynabead coupling. After elution, add 50 % glycerol for storage or dialyze or desalt into the buffer of choice.

3.6 Affinity Capture of RNPs Using Conjugated Magnetic Medium

For a comprehensive review of considerations affecting expression systems, epitope tagging, and affinity medium choice see [45]. In brief, we find that Dynabeads, when conjugated to high-quality antibodies, provide for excellent quality recovery of endogenous protein complexes from human cells [37, 38]. We have successfully applied this approach to L1 RNPs [6]. For purifying 3× Flag-tagged constructs, α-Flag Dynabeads are necessary, prepared as above. When combined with neodymium magnet racks, antibody-conjugated magnetic medium (beads) is rapidly separated from the buffer and immobilized on the side of the tube. This allows near-complete aspiration of buffer without the risk of aspirating the beads.

1. Prepare clarified cell extracts:
   (a) Weigh out 200 mg of cell powder into a microcentrifuge tube—hold on LN_2 (*see* **Note 53**).
      i. Repeat (a) for as many purifications as you will carry out.
      ii. Multiple purifications can be pooled after elution if larger scale is required.
   (b) Move the tubes to room temperature for 1–2 min (*see* **Note 54**).

(c) Add 800 μL of extraction solution (*see* **Note 55**) (20 mM Na-HEPES pH 7.4, 500 mM NaCl, 1 % v/v Triton X-100; plus protease inhibitors) to each tube, vortex for ~30 s until powders are resuspended, and then place the crude extracts on ice. Some membrane aggregates may be observed (*see* **Note 56**).

(d) Sonicate each tube with a micro-tip probe on a low-power setting using 2 × 2 s pulses. Membrane aggregates should no longer be visible (*see* **Note 57**).

(e) Centrifuge for 10 min at full speed (20–30,000 RCF) in a refrigerated microcentrifuge at 4 °C.

i. During this step the affinity medium can be pre-washed (**step 2a**).

(f) Remove supernatant—this is your clarified extract—and add to the tube containing α-Flag Dynabeads (**step 2b**).

i. Set a fraction aside before combining with beads to compare pre- and post-bead binding (**step 2ci**) in order to assess the efficacy of the affinity capture.

2. Affinity capture:

(a) Prepare beads:

i. Pipette 20 μL of α-Flag Dynabeads slurry into a 1.5 mL microcentrifuge tube (*see* **Note 58**).

ii. Repeat for each affinity purification to be carried out.

iii. Wash the beads twice with 500 μL of extraction solution.

iv. Remove the supernatant, and hold the beads on ice until needed.

(b) Combine the clarified extract (**step 1f**) with the beads.

i. Incubate at 4 °C for 30 min (*see* **Note 59**).

(c) Separate beads on a magnetic separator. Set a fraction aside to compare with input (**step 1fi**), and aspirate the remainder.

(d) Wash the beads with 1 mL of extraction solution and then remove the supernatant. Wash protocol for beads (used throughout):

i. Add buffer, and vortex at full power for 2–3 s.

ii. Pulse-spin in a benchtop microcentrifuge to remove any magnetic beads from the cap.

iii. Separate beads on a magnetic separator, and remove buffer using a vacuum aspirator.

(e) Resuspend the beads in 1 mL of extraction solution, transfer to a fresh microcentrifuge tube, and then remove the supernatant (*see* **Note 60**).

(f) Wash again with 1 mL of extraction solution.

(g) Elute the L1 RNPs from the beads with 26 μL of 1 mg/mL 3× Flag peptide in extraction solution (native) or using 1× SDS-PAGE loading buffer without reducing agent (*see* **Note 61**).

i. Native elution: Incubate for 15–30 min at room temperature with gentle agitation (just enough to mix and suspend the beads).

ii. Denaturing elution: Incubate for 5–10 min at 70 °C with moderate agitation (*see* **Note 62**).

iii. If native elution is used, remove the eluate and then perform a second denaturing elution to assess the efficacy of the native elution.

3. Natively eluted samples can be carried forward to other assays (see LEAP and RT-PCR below) and/or subsequently prepared for SDS-PAGE (*see* **Notes 63–65**).

4. For samples to be analyzed by mass spectrometry, reduce and alkylate with iodoacetamide:

(a) Add SDS-PAGE loading dye to 1× and DTT to 20 mM (*see* **Note 66**).

(b) Incubate for 10 min at 70 °C.

(c) Cool to room temperature.

(d) Add iodoacetamide to 0.1 M and incubate in the dark at room temp for 30 min.

(e) Load directly on a gel.

3.7 Sample Preparation for Mass Spectrometry

Proper preparation of samples is critical for mass spectrometry as a number of interfering species can reduce sensitivity and compromise protein identification (for discussion and advice, see [45]). For identification of the most prominent species, readily observed by standard protein staining techniques (reviewed in [46, 47]), we excise regions of the gel containing stained protein bands and use MALDI-MS peptide mass fingerprinting (PMF, [48, 49]; example protocols that we use can be found in the supplement of [38]). For more sensitive detection and identification of the complement of proteins within the sample we use liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) approaches (reviewed in [50, 51]). If proteins are first metabolically labeled with stable isotopes prior to affinity capture, then isotopic differentiation of interactions as random or targeted (I-DIRT) can be implemented. This MS-based analysis provides statistical discrimination between interactors likely to have originated in vivo and those likely to be in vitro artifacts, and can be accomplished by modifying the procedures outlined in this chapter [6, 52, 53]. Whether MALDI-MS or LC-MS/MS will be used, we prefer gel-based peptide sample

work-up [54]. Because different MS-based analytical approaches may require different sample work-up procedures, we recommend adopting appropriate procedures based on the preferences of your proteomics core facility or collaborator.

3.8 LEAP (L1 Element Amplification Protocol)

This protocol uses ORF2p reverse transcriptase and an anchor primer to reverse transcribe bound RNA [23]. cDNA is then amplified by PCR for visualization on a gel or quantification by real-time PCR. A boiled sample is used for a negative control. cDNA is made with commercial reverse transcriptase as a positive control.

1. Thaw RNPs on ice. Clean bench and work area using RNAseZap or similar. Open a clean box of pipet tips.
2. Prepare sufficient LEAP reaction mix for twice the number of samples (see recipe, Subheading 3).
3. Prepare LEAP + SuperScript reaction mixture:
   (a) Pipet half of the LEAP buffer into a clean tube.
   (b) Add SuperScript III Reverse Transcriptase (0.25 μL per reaction, 50 U), and mix well.
4. For the negative control, add 5 μL of one RNP sample to a clean tube. Boil for 5 min at 100 °C.
5. Aliquot LEAP and LEAP + SuperScript reaction mixtures into reaction tubes.
6. Add 2 μL of each RNP to both LEAP and LEAP + SuperScript mixture. Do not forget to add the boiled negative control.
7. Incubate LEAP reactions for 1 h at 37 °C. Incubate LEAP + SuperScript reactions for 1 h at 50 °C (*see* **Note 67**).
8. Optional pause point: Snap-freeze LEAP products.
9. Amplify LEAP products for visualization and/or sequencing:
   (a) Use 1 μL LEAP product in a 50 μL PCR reaction using FastStart Taq DNA Polymerase with primers JB11564 and JB14057.
10. Measure LEAP products by qRT-PCR using a StepOne Plus instrument or similar:
    (a) Use 0.5 μL of each LEAP product in triplicate 20 μL reactions with SYBR Green 2× master mix.
    (b) Measure the ORF1 region of the L1 RNA with primers JB13415 and JB13416 (*see* **Note 68**).
    (c) Measure the ORF2 region of the L1 RNA with primers JB13417 and JB13418.

3.9 RNA Isolation and qRT-PCR of L1 RNPs

Total RNA is isolated from L1 RNPs after spiking in purified GFP-transfected HEK-293T RNA (*see* **Note 4**). This serves as both a carrier and as an internal control for normalization. Alternatively, in vitro-transcribed GFP mRNA may be used along with glycogen

as a carrier. A practical review of qRT-PCR methods can be found in [55].

1. Aliquot 2 μg purified GFP-transfected control RNA into each tube.
2. Add 10 μL of each RNP sample, and mix well.
3. Purify RNA using TRIzol Reagent according to the manufacturer's protocol.
4. Resuspend RNA in 20 μL RNase-free water.
5. Quantify by qRT-PCR as in Subheading 3.8, **step 10**, using 0.5 μL resuspended RNA in triplicate. Primers for GFP (JB13766 and JB13767) are used for normalization of each sample.

4 Notes

1. This affinity-purified version performs as well or better than the non-affinity-purified F3165, and at the time of writing is approximately half the cost.
2. The Dynamag 15 rack is useful for couplings starting with 300 mg Dynabeads. We often do smaller test couplings with 60 mg Dynabeads, which can be done with 2 mL tubes. To reduce cost, high-quality neodymium magnets from MAGCRAFT are widely distributed and available in a variety of shapes. These can be fitted into homemade racks or attached to tubes using rubber bands; arc-shaped magnets are particularly useful with rubber bands.
3. The pH of 10× PBS is between 6.6 and 6.7 at 10× but is 7.4 when diluted to 1×.
4. Buffer components for affinity capture and LEAP should be RNase free, and handled with precautions for cleanliness appropriate for RNA work. This includes buffers used for dissolution of 3× Flag peptide, etc..
5. We transfect HEK-293TLD cells with pCAG-eGFP (Addgene # 11150) and purify total RNA using an RNeasy mini kit (Qiagen).
6. Life Technologies reports that Freestyle 293 provides serum-free growth of their engineered HEK-293-F cells. In our experience with other HEK-293 and HEK-293T cells, 1 % tet-free FBS adds little cost to the medium and increases reliability. Similarly, supplemental l-glutamine, added fresh, provides more reliable growth than their GlutaMax alternative alone. We make a 50:50 mix of serum and 200 mM l-glutamine, filter, and add 20 mL per liter.

7. We have been unable to establish an effective protocol for puromycin selection after suspension transfection.
8. Always autoclave with loose bottle caps. The tops of the bottles can be covered in foil for additional protection. After the autoclave finishes, transfer bottles to a culture hood to cool, and then tighten caps for storage.
9. If any bottle is found to be contaminated, all bottles in the incubator must be checked for signs of contamination.
10. Until a suspension line is well established or after steps that can be toxic to cells, we find a minimum density of 0.5 million/mL is safer than 0.2 million/mL.
11. Do not directly transfer from the serological pipet to the hemocytometer.
12. Never use a micropipettor in suspension bottles: this risks contamination.
13. This ratio should be optimized for different cell lines and DNA preparation methods.
14. After column purification, precipitate with isopropanol and wash with ethanol. DNA prepared with their precipitator modules has been less effective in our experience.
15. To save time, hybridoma SFM aliquots can be left at room temperature overnight.
16. For small numbers of transfections, prepare DNA-PEI complex in conical tubes. For larger volumes, use a glass bottle.
17. ORF2p expression peaks 24 h after induction and falls thereafter. ORF1p expression peaks at ~18 h after induction and is constant for at least 4–5 days. Harvest time should be optimized for your protein of interest.
18. We have found that with episomal pCEP4-based plasmids, a 1:3 "split-back" minimally reduces per-cell yield but allows the use of threefold less DNA per gram WCW.
19. We find that transfection efficiency is higher in 6-well wells than larger scales, so we transfect a number of wells and then pool the cells later.
20. If protease is used, quench with serum-containing media and centrifuge for 5 min at 200 RCF to remove the protease.
21. Recipe for suspension medium is as described above, but when following the protocol for adherent transfection, selection, and adaptation to suspension it may include antibiotics.
22. Cells can be adapted into higher puromycin concentrations of 2–10 μg/mL, resulting in higher per-cell expression but slower growth and longer time needed for adaptation.
23. Suspension cells are more sensitive to puromycin than adherent cells.

24. It can be helpful to perform a test induction at this point. Plate 0.5 million cells in each of the two 6-well wells. Add 1 μg/mL doxycycline to induce one. Lyse 24 h later for western blotting and freeze lysate for later comparison.
25. Note that this 90:10 medium contains ~2 % FBS.
26. Once cells stably transfected with a construct of interest are growing in suspension, it is advisable to freeze aliquots for later use. We freeze 20 million cells per vial in suspension medium supplemented with 5 % DMSO and 20 % FBS.
27. A "BB" is a small round steel ball, like those used in toy guns. Cells frozen in this way form spheroid shapes (and globules of spheroids).
28. Pellets should appear approximately uniform. If centrifuged too hard, cells will be crushed, forming two different-colored layers.
29. Depending on the syringes used, it may be necessary to trim the finger grips so the syringe fits inside the tube. Thirty milliliter syringes are taller than 50 mL conical tubes. Make sure that these have enough clearance in your swinging bucket centrifuge before adding cells.
30. In order to thoroughly remove the liquid, we commonly suck off the very top layer of cells in order to remove the PBS. We use a vacuum aspirator and glass Pasteur pipet.
31. Use care and best practices in handling liquid nitrogen. Appropriate protective gear and goggles should be used to prevent injury.
32. Each tube will hold approximately 15–20 g BB's. For very large samples, we use polypropylene bottles.
33. A pair of pliers may be needed to remove the luer-lock syringe cap after centrifugation.
34. Storage with a punched cap is acceptable for a few days; however frost will form on cells over longer periods.
35. 1 g WCW is approximately the minimum amount of cells for grinding. A small amount of material (up to ~500 mg) is lost on the surface of the jar and balls, so practically we aim to produce at least 1.5–3 g WCW.
36. The custom PTFE insulators are not required for this protocol [38]. However, we find that grinding is greatly aided by the inclusion of LN within the jar ("wet grinding"). The insulators prevent warming of the jar, evaporation of the nitrogen, and pressure buildup, resulting in faster, more reliable grinds. To have insulators made at the Rockefeller University machine shop, contact the Rout Lab.
37. All tools for grinding should be chilled during use.

38. We use two containers: a Styrofoam box filled with enough N2 to completely submerge the jar, and a pan for sample prep and intermediate cooling steps in which the jar is not completely submerged. When grinding multiple samples, we put a tube rack in the Styrofoam box for storage of BB's and grindate and cover the box with its lid.
39. Clamping force should be firm, but not excessive such that removal of the jar becomes a problem.
40. Grinding should make a distinct clunking noise as the balls collide. This noise may stop at some point during a rotation, but should resume when rotation is reversed. If these sounds are not heard, inspect between cycles to make sure that grinding is occurring.
41. Releasing too quickly can cause rapid depressurization and loss of grindate. A controlled release allows gentle depressurization, which can be heard as a gentle hiss.
42. Grindate can be stored at −80 °C essentially indefinitely, without affecting performance.
43. Protein recovery can be verified by Bradford assay or SDS-PAGE with Coomassie, comparing input and output. For Bradford use gamma globulin as the standard to get an accurate concentration.
44. This results in ~2.2 mL final volume, and a slurry of approximately ~10–15 % by volume.
45. Measurements of coupling yield were done using rabbit polyclonal IgG. The apparent capacity of the beads may vary depending upon the coupling conditions, including the concentration of salt, ammonium sulfate, pH, and temperature of coupling.
46. With anti-Flag M2, beads conjugated at 8 μg antibody/mg Dynabeads do not perform as well as beads conjugated at 10 μg/mg. There is only a marginal improvement in going from 10 to 12.5 μg/mL; we use 10 μg/mg as a cost-effective compromise.
47. Sometimes resuspension requires more volume. Simply remove the buffer from the beads using a magnetic separator and wash the bottle with fresh buffer.
48. The addition of BSA increases the long-term stability of M2-Flag antibody-conjugated Dynabeads.
49. 30 °C is also effective.
50. With proper storage, coupled Dynabeads may be used without loss of performance for >1 year. Alternatively, if the beads will be completely consumed within ~8 weeks, storage at 4 °C is suitable—resuspend with PBS, 0.5 mg/mL BSA, and 0.02 % sodium azide and store at 4 °C.

51. Depending on the antibody species and subtype, protein A may be more appropriate than protein G.
52. A variety of Protein G preparations, including magnetic, are commercially available. At this scale, magnetic medium is much more expensive.
53. Use a small Styrofoam rack on a microbalance and pre-chill tubes and weighing instruments on LN2. We have found that this is easiest using inexpensive small stainless steel measuring spoons designed for culinary use.
54. Allowing the tube to briefly warm prevents extraction solution from flash freezing on the side of the tube.
55. Determination of the optimal extraction solution for each complex is critical but beyond the scope of this chapter. For more information see LaCava et al. (45).
56. Hold tubes on ice between each subsequent manipulation—working at room temperature is otherwise acceptable.
57. The power should be adjusted such that the minimum amount of energy is used that will disperse the aggregates. On a Branson Sonifier with Microtip, this is power setting 3.
58. The ratio of beads to lysate can be optimized. For α-ORF1p pullouts 50 μL Dynabeads were needed to deplete the ORF1p from extracts. For α-ORF2p pullouts, 10 μL was sufficient and background increased with larger amounts.
59. This time can be optimized. For α-ORF1p pullouts, 5 min was as effective as 30 min. For α-ORF2p pullouts using α-Flag Dynabeads, 1 h was more effective than 30 min.
60. We find that transfer to a fresh tube at this step reduces background because some protein nonspecifically sticks to the tube.
61. Reducing agent is omitted to reduce the release of IgG from the beads. It should be added after separation from the magnetic medium, before denaturation for SDS-PAGE.
62. We use a Thermomixer (Eppendorf) at full speed.
63. The advantage of native elution, even if SDS-PAGE is the next step, is its tendency to release only specific interactors of the tagged protein, reducing nonspecific contamination.
64. Regardless of the elution method, all samples should be reduced and alkylated prior to loading on the gel to maximize sensitive detection of cysteine-containing peptides during subsequent MS.
65. For storage of purified RNPs, for example for future LEAP, we dilute 1:1 with 50 % glycerol (25 % final), flash freeze with N2, and store at −80 °C.
66. This is 40 % of the typical 50 mM DTT concentration in reducing SDS-PAGE loading dye.

67. Incubation at 50 °C favors the SuperScript III reaction over the ORF2p reaction. At 37 °C both reactions occur simultaneously.
68. These primers are specific for ORFeusHS; appropriate primers should be chosen for other L1 elements.

Acknowledgements

We thank Dan Leahy, Jennifer Kavran, and Yana Li for help with suspension cell culture. This work was supported in part by NIH grant U54GM103511 to MPR, grant 5P50GM107632 to JDB, KHB, and MPR, U54GM103520 to JDB, R01CA163705 and R01GM103999 to KHB, and US DoD grant OC120390 to KHB.

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Exhibit P

Adeno-Associated Virus Production, Purification, and Titering

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Adeno-associated virus (AAV) vectors are exemplary tools for studying gene function *in vivo* and are particularly favorable for transferring genes of interest into brain tissues. They have shown great promise as a gene therapy vector for preclinical and clinical applications. However, the ability to use this tool is often hampered because the viruses themselves are not readily available. Many methods have been developed for AAV production. Here, we describe a simple method for small- to medium-scale (10^{12}-10^{13} viral particles) production of AAV based on Polyethylenimine Max (PEI Max)–mediated triple transfection of HEK 293 cells and purification with iodixanol gradient ultracentrifugation. These methods will provide users with ample material of sufficient quality for performing *in vivo* gene transfer. © 2018 by John Wiley & Sons, Inc.

Keywords: adeno-associated virus • iodixanol gradient ultracentrifuge • PEI Max transfection

How to cite this article:
Chen, Y. H., Keiser, M. S., & Davidson, B. L. (2018). Adeno-associated virus production, purification, and titering. *Current Protocols in Mouse Biology*, *8,* e56. doi: 10.1002/cpmo.56

INTRODUCTION

Adeno-associated virus (AAV) is a commonly used viral vector for biomedical research and gene therapy. It exhibits limited pathogenicity or cytotoxicity at all but the very highest doses, which are rarely required for gene expression. AAV genomes can exist stably in an episomal state with a low rate of genomic integration, which decreases the risk of insertional mutagenesis. Wild-type AAV belongs to the parvovirus family and is composed of a 4.7-kb single-stranded DNA encapsulated in a non-enveloped capsid. The viral genome encodes three genes (*rep*, *cap*, and *aap*) flanked by inverted terminal repeats (ITRs) that function as the viral origin of replication and packaging signal. The *rep* gene encodes proteins for viral genome replication, transcriptional regulation, and packaging. The *cap* gene encodes three structural proteins (VP 1-3) that assemble to make the viral capsid. The *aap* gene encodes the assembly-activating protein (AAP) for viral assembly. The AAV vectors we commonly use are recombinant AAVs in which the *rep*, *cap*, and *aap* genes are replaced by a transgene expression cassette.

Here we provide detailed protocols for AAV production, purification, and titering. The Basic Protocol describes a method for AAV production by triple transfection of three plasmids into HEK 293 cells. The *cis*-plasmid encodes the transgene flanked by ITRs. Two *trans*-plasmids provide the AAV *rep, cap*, and *aap* genes for AAV packaging and adenovirus genes for adenovirus helper. Support Protocol 1 describes a method for AAV

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Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmo.56

purification by iodixanol gradient ultracentrifugation. Support Protocol 2 provides a method for AAV titer evaluation by quantitative PCR.

CAUTION: All equipment and reagents dedicated to tissue culture and vector purification should be sterile, and proper sterile technique should be used accordingly.

BASIC PROTOCOL

AAV PRODUCTION IN HEK 293 CELLS

This protocol describes the production of crude AAV lysate by PEI Max–mediated triple transfection of HEK 293 cells.

Materials

HEK 293 cells (ATCC, CRL-1573)
0.05% trypsin/EDTA (Thermo Fisher, 25300054)
DMEM-5 and DMEM-10 (see recipe)
Opti-MEM, Reduced Serum (Thermo Fisher, 31985062)
1 mg/ml PEI Max solution (see recipe)
Plasmids:
  Adenovirus helper plasmid, pAd helper (Takara Bio, 6234)
  Rep/Cap packaging plasmid, pAAV.RepCap (Takara Bio, 6234)
  Transgene plasmid, pTransgene (developed in-house or commercially available, e.g., Addgene)
Polyethylene glycol (PEG) 8000 (Research Products International, 48080)
NaCl (Fisher Chemical, S271-10)
Dry ice/ethanol bath
PBS-MK (see recipe)
250 U/μl benzonase (Sigma-Aldrich, E1014)

145-mm cell culture plates (Greiner Bio, 639160)
37°C, 5% CO_2 incubator
Hemocytometer
Light and fluorescent microscopes
15- and 50-ml polypropylene centrifuge tubes
Sterile cell scraper (Fisher Scientific, 08-100-242)
500-ml bottler-top filter with 0.22-μm PES membrane
37°C water bath

Seed cells

1. Prepare four 145-mm plates of HEK 293 cells at a passage lower than 25 and at 80-90% confluence.

   Four 145-mm plates of freshly grown cells will be combined to seed 10 plates for transfection. Scale up as needed.

2. Warm 0.05% trypsin/EDTA and DMEM-10 to 37°C.

3. Aspirate culture medium, add 6 ml of 0.05% trypsin/EDTA to each plate, and incubate in a 37°C, 5% CO_2 incubator for 6-8 min.

4. Add 4 ml DMEM-10 to neutralize the trypsin and gently pipet up and down 5-10 times in the plate to disperse cells into a single-cell suspension.

5. Transfer the suspension from four plates into a single 50-ml polypropylene centrifuge tube, then centrifuge 5 min at 300 × *g*, 4°C.

6. Aspirate supernatant and resuspend pellet in 30 ml prewarmed DMEM-10.

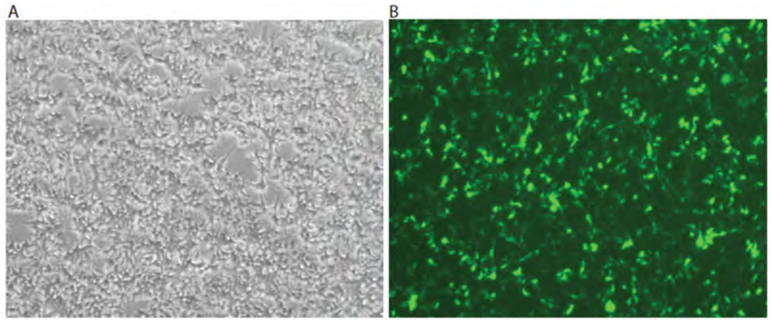

Figure 1 HEK 293 cells for rAAV production. (**A**) HEK 293 cells at ~80% confluence for plasmid transfection. (**B**) eGFP expression in HEK 293 cells 24 hr after transfection.

7. Count cells with a hemocytometer and adjust concentration to 7×10^5 cells/ml with additional DMEM-10.

8. Seed 20 ml cell suspension (1.4×10^7 cells) in a 145-mm plate and place in the incubator for 24 hr.

   OPTIONAL: *HEK 293 cells can be seeded at a lower density by adding 20 ml of a 2.5×10^5 cells/ml suspension per plate. These plates will be ready for transfection after 48 hr. The advantage of this perfomance is that more plates can be seeded if higher viral yield is required.*

Transfect cells

9. Use a microscope to check the confluency of the HEK 293 cells.

   The optimal confluency for transfection is 80% (Fig. 1A).

10. Warm Opti-MEM and 1 mg/ml PEI Max to room temperature.

11. In a 50-ml tube, prepare enough plasmid mixture for ten plates by adding the following amounts of plasmid to Opti-MEM to give a final volume 3.75 ml. Mix well.

    267.7 µg pAd helper
    189.2 µg pAAV.RepCap
    143.1 µg pTransgene

    To calculate the individual amount of each plasmid/plate, operate on the assumption that 60 µg of total DNA per plate with a molar ratio of 1:1:1 for the three plasmids will be transfected. The combined length of total plasmid DNA of all three plasmids is up to 26 kb (pAd helper is 11.6 kb; pAAV.RepCap is 8.2 kb; pTransgene is 6.2 kb). The calculations to derive the required moles of DNA for each plasmid is below:

    moles of dsDNA (mol) = mass of dsDNA (g)/([length of dsDNA (bp) × 617.96 g/mol] + 36.04 g/mol)

    = 0.060 g/([26,000 bp × 617.69 g/mol] + 36.04 g/mol)

    = 3.734 pmol

    where 617.96 g/mol is the average molecular weight of a base pair, excluding the water molecule removed during polymerization, and 36.04 g/mol accounts for the 2-OH and 2-H added back to the ends.

    Then, using 3.734 pmol DNA for each plasmid, the required µg of each plasmid is calculated as:

    mass of dsDNA (g) = moles of dsDNA (mol) × ([length of dsDNA (bp) × 617.96 g/mol] + 36.04 g/mol)

For example:

$$\text{mass of pAd helper (g)} = 3.734\ \text{pmol} \times ([11{,}600\ \text{bp} \times 617.96\ \text{g/mol}] + 36.04\ \text{g/mol})$$

$$= 26.77\ \mu g \text{ per plate}$$

An online calculator is available at https://nebiocalculator.neb.com/#!/dsdnaamt.

12. In another 50-ml tube, add 1.2 ml of 1 mg/ml PEI Max to 2.55 ml Opti-MEM for a final volume of 3.75 ml (a ratio of 2 µg PEI Max to 1 µg DNA). Mix well.

13. Add PEI Max/Opti-MEM to the plasmid/Opti-MEM mixture, pipet up and down several times, and incubate at room temperature for 15 min.

14. Pipet up and down several more times, then add 750 µl transfection mixture to each plate of cells. Transfer plates to the incubator for 24 hr and monitor transfection efficiency using a fluorescent microscope if pTransgene expresses a fluorescent protein such as eGFP (Fig. 1B).

    In a successful transfection, >70% of cells exhibit eGFP expression.

15. Change medium 24 hr after transfection. Aspirate culture medium and transfection mixture, then slowly add 20 ml prewarmed DMEM-5 per plate. Be careful not to disrupt the cell monolayer.

16. Incubate plates for an additional 48 hr.

Harvest virus

17. Harvest cells and medium 72 hr post-transfection. Draw off 5 ml medium from the plate and set aside in a 15-ml conical tube. Lift cells off the plate using a sterile cell scraper and transfer cells and medium to 50-ml tubes. Rinse plate with the 5 ml previously set aside.

18. Pool all cells and medium, then centrifuge 10 min at 300 × *g* (4°C).

19. Filter supernatant with a 500-ml bottle-top filter with a 0.22-µm PES membrane. Keep the cell pellet on ice.

20. For supernatant:

    a. Add 10 g PEG 8000 and 5.8 g NaCl per 100 ml supernatant and stir at 4°C until PEG and NaCl are completely dissolved (~1 hr).
    b. Store at 4°C overnight.
    c. Centrifuge 30 min at 5000 × *g*, 4°C.
    d. Discard supernatant and resuspend pellet (containing virus and protein) in 5 ml PBS-MK (0.5 ml per dish).

21. For cell pellet:

    a. Resuspend cell pellet in 10 ml PBS-MK (1 ml per plate).
    b. To lyse cells, freeze the suspension in a dry ice/ethanol bath, then thaw in a 37°C water bath, and vortex briefly (5 sec). Repeat for a total of three cycles.

22. Combine the thawed cell lysate and resuspended virus from supernatant in a 50-ml tube.

23. Add benzonase to a final concentration of 50 U/ml (1 µl per 5 ml). Incubate at 37°C for 30 min.

24. Centrifuge 20 min at 3000 × *g*, 4°C to remove cellular and protein debris.

25. Transfer the clarified supernatant to a new tube.

This should yield ∼14 ml crude AAV lysate, which can be stored at 4°C overnight before purification by iodixanol gradient ultracentrifugation.

AAV PURIFICATION WITH IODIXANOL GRADIENT ULTRACENTRIFUGATION

SUPPORT PROTOCOL 1

This protocol is for the production of purified AAV vectors from crude AAV lysate prepared in the Basic Protocol.

Materials

15%, 25%, and 40% iodixanol solutions (see recipe)
60% iodixanol solution (Opti-Prep, Sigma-Aldrich, D1556)
Clarified AAV supernatant (see Basic Protocol)
70% (v/v) ethanol
Vector stock buffer (see recipe)

39-ml Ultra-Clear QuickSeal tubes (25 × 89 mm, Beckman Coulter, 344326)
10-ml syringes
14-G, 4-inch pipetting needles with 90° blunt ends (Sigma-Aldrich, CAD7941)
Beckman Coulter ultracentrifuge with Type 70 Ti rotor
1.5-ml microcentrifuge tubes
Stand with clamp
18-G needles
Vivaspin 6 centrifugal concentrator, MWCO 100 K (Vivaproducts, VS0641)

Additional reagents and equipment for SDS-PAGE and Coomassie blue staining

Prepare gradient

1. Load the following iodixanol solutions in order into a 39-ml QuickSeal tube using a 10-ml syringe with a 14-G, 4-inch pipetting needle:

   9 ml 15% iodixanol
   6 ml 25% iodixanol
   5 ml 40% iodixanol
   5 ml 60% iodixanol.

   Place the needle in the bottom of the tube, then slowly load the solutions at a rate of 2 ml/min, being careful to avoid introducing bubbles.

   By underlaying each new solution, the 15% solution will be on the top of the gradient and the 60% solution on the bottom. A sharp layer will form between gradient interfaces under bright light (Fig. 2A).

2. Carefully overlay the clarified supernatant on top of the gradient using a 10-ml syringe with a 14-G, 4-inch pipetting needle (Fig. 2B). Touch the needle tip to the surface of the 15% iodixanol solution and slowly load the sample at ∼2 ml/min. Avoid introducing bubbles.

3. Seal the QuickSeal tubes. Draw a line with a permanent marker on the outside of the tube at each interface.

   The interfaces can become obscured after centrifugation (Fig. 2C).

4. Centrifuge 1 hr at 350,000 × g, 18°C using a Type 70 Ti rotor with a slow acceleration/deceleration program.

5. Take the tubes out of the rotor and place them in a stable rack. Be careful not disturb or mix the gradient with excessive motion.

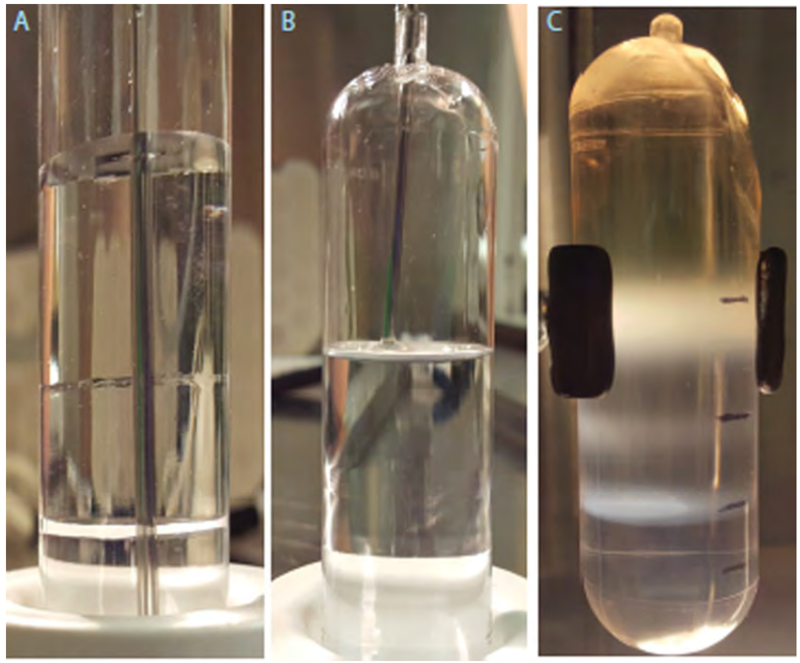

Figure 2 Iodixanol gradient ultracentrifugation for rAAV purification. (**A**) Loading the iodixanol gradient with a long needle. (**B**) Loading the rAAV crude lysate on top of the iodixanol gradient. (**C**) Iodixanol gradient after ultracentrifugation. Black lines indicate the interface between different iodixanol gradients.

Collect virus

6. Prepare fifteen 1.5-ml microcentrifuge tubes. Draw a sharp line on the side of the tubes at 0.75 ml to help guide fraction collection.
7. Fix the QuickSeal tube onto a stand with a clamp, then swab the tube with 70% ethanol.
8. Puncture the top of the QuickSeal tube with an 18-G needle.
9. Puncture a small hole at the bottom of the QuickSeal tube with an 18-G needle and collect fifteen 0.75-ml fractions per tube.

   The initial flow rate will be quite slow, as the highest density layer is collected first. The flow rate will increase as the density decreases.

10. Analyze the contaminating protein and virus in the fractions by protein gel electrophoresis and Coomassie blue staining.

    Only the cleanest fractions with VP1-3 bands, which represent the three AAV capsid proteins, will be pooled (Fig. 3).

    Alternative: Puncture the QuickSeal tube slightly below the 40-60% interface with an 18-G needle attached to a 10-ml syringe with the bevel facing up. Collect 4 ml. Take care to avoid collecting the protein-rich material at the 40-25% interface (Fig. 2C).

Perform diafiltration and concentration

11. Add 5 ml vector stock buffer to a Vivaspin 6 concentrator and centrifuge 5 min at 1800 × *g*, 4°C.
12. Dilute the virus prep three-fold with virus stock buffer.
13. Load on the Vivaspin 6 concentrator and centrifuge 5 min at 1800 × *g*, 4°C. Mix solution by pipetting up and down five times in the top chamber.

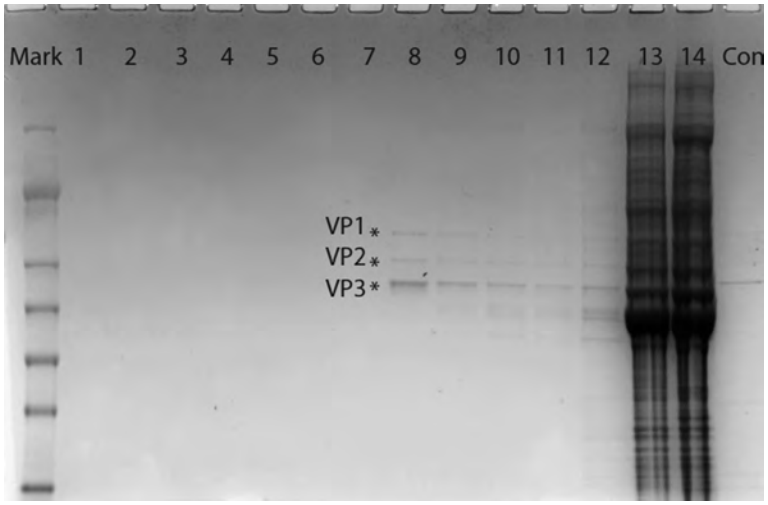

Figure 3 Example of a Commassie blue–stained 4-12% SDS-PAGE gel after electrophoresis of 14 gradient fractions collected from the centrifuge tube. Asterisks indicate AAV capsids VP1, VP2, and VP3. The control is 10^{10} physical particles of purified rAAV.

14. Repeat until the volume of solution in the top chamber is ~0.5-1 ml.
15. Repeat with the rest of virus as above.
16. Add 5 ml fresh virus stock buffer and spin as above.
17. Repeat step 16 two more times and concentrate the virus to the final volume as needed (200-500 µl recommended).
18. Set aside 10 µl virus for titering (see Support Protocol 2). Keep at 4°C for up to 1 week.
19. Aliquot remaining virus at 25 to 50 µl and freeze at −80°C for long-term storage 2 years; avoid repeated freeze-thaw cycles.

TITERING AAV PARTICLES BY QUANTIFICATION PCR (Q-PCR)

SUPPORT PROTOCOL 2

This protocol is for the titering AAV genomic particles in purified AAV vectors by Q-PCR.

Materials

pTransgene plasmid to be used as Q-PCR standard
Suitable restriction enzyme (e.g., XbaI) and buffer (e.g., Cutsmart)
Ultrapure distilled H_2O (Invitrogen, 10977015)
PureLink PCR purification kit (Thermo Fisher, K310001)
Qubit dsDNA HS assay kit (Thermo Fisher, Q32854)
Purified AAV (see Support Protocol 1)
DNase (Thermo Fisher, AM 2238) with 10× DNase buffer
0.5 M EDTA, pH 8.0 (Corning, 46034Cl)
TaqMan universal master mix II (Thermo Fisher, 4440040)
Q-PCR primer/probe (designed by individual laboratory or commercially available)

Sequence analysis software (e.g., Sequencher, MacVector, Lasergene, Geneious, or Snapgene)
1.5-ml microcentrifuge tubes
384-well Q-PCR plate

Real-time thermal cycler (Bio-Rad, CFX 384)

Prepare Q-PCR standards

1. Obtain a plasmid with the same gene or promoter as pTransgene used in AAV production.

2. Look for unique restriction sites in pTransgene using sequence analysis software (such as Sequencher, MacVector, Lasergene, Geneious, or Snapgene). Linearize the plasmid with a suitable restriction enzyme that is outside of the primer probe region. For the example pTransgene in the Basic Protocol, we use XbaI in the following reaction:

   3 µl 1 µg/µl pTransgene
   5 µl Cutsmart buffer
   2 µl XbaI restriction enzyme
   40 µl H_2O.

   Incubate at 37°C for 5 hr.

3. Purify the digest product with a Purelink PCR purification kit as per kit instructions.

4. Quantify the linearized plasmid DNA concentration by Qubit dsDNA HS assay kit.

5. Make 1 ml of plasmid stock at 1×10^{11} copies/ml. First, convert the unit of DNA concentration from ng/µl to copies/µl using the Mass-to-Mole function of the online calculator *https://nebiocalculator.neb.com/#!/dsdnaamt*.

   For a plasmid size of 5210 bp and a linearized plasmid DNA concentration of 15 ng/µl, the calculations are:

$$\begin{aligned}\text{moles dsDNA (mol)} &= \text{mass of dsDNA (g)/([length of dsDNA (bp)} \times 617.96\text{ g/mol]} + 36.04\text{ g/mol)}\\ &= 15 \times 10^{-9}\text{ g/([5210 bp} \times 617.96\text{ g/mol]} + 36.04\text{ g/mol)}\\ &= 4.741 \times 10^{-15}\text{ mol}\\ \text{DNA copy number} &= \text{moles of dsDNA} \times 6.022\times 10^{23}\text{ molecules/mol}\\ &= 4.741 \times 10^{-15}\text{ mol} \times 6.022\times 10^{23}\text{ molecules/mol}\\ &= 2.855 \times 10^{9}\text{ copies/}\mu\text{l}\end{aligned}$$

   Here, 617.96 g/mol is the average molecular weight of a base pair, excluding the water molecule removed during polymerization; 36.04 g/mol accounts for the 2-OH and 2-H added back to the ends; and 6.022×10^{23} is Avagadro's number.

   Dilute in 1 ml H_2O as needed for 1×10^{11} copies/ml.

6. Make a serial dilution of this stock to generate solutions with 1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7}, 1×10^{6} and 1×10^{5} copies/ml. Aliquot and keep up to 6 months at –20°C.

Treat AAV with DNase and dilute

7. Prepare the DNase reaction in a 1.5-ml tube:

   2 µl purified virus
   2 µl DNase buffer
   1 µl DNase
   15 µl H_2O.

   Incubate at 37°C for 30 min.

8. Inactivate DNase by adding 1 μl of 0.5 M EDTA, pH 8.0, and incubating at 75°C for 10 min.
9. Centrifuge 30 sec at 12,000 × *g*.
10. Make 1:1000 and 1:10,000 virus dilutions. For the 1:1000 dilution, add 2 μl DNase-treated AAV (already diluted 1:10.5) to 188.5 μl H_2O. For the 1:10,000 dilution, add 10 μl of the 1:1000 dilution to 90 μl H_2O.

Perform Q-PCR and determine titer

11. Set up Q-PCR reaction. Prepare and run each sample in triplicate (the serial dilution standard from 1×10^{10} to 1×10^5 copies/ml from step 5 and the two virus dilutions from step 10). In each well of a 384-well Q-PCR plate, add:

    5 μl Taqman master mix
    0.5 μl 20× primer/probe
    4.5 μl sample

12. Run the Q-PCR program in a real-time thermal cycler:

    Step 1: 95°C for 10 min
    Step 2: 95°C for 10 sec
    Step 3: 60°C for 1 min
    Repeat steps 2 and 3 for 39 cycles

13. Use the standard curve to predict the titer of the vector. Make sure to account for the dilution factor and the single-stranded nature of the AAV vector genome using the following formula:

    $$\text{Virus titer} = \text{quantification result of each dilution} \times \text{dilution factor} \times 2$$

    Determine the final AAV titer as the average of the two dilutions.

REAGENTS AND SOLUTIONS

DMEM-5 and DMEM-10

500 ml DMEM, High Glucose (Thermo Fisher, 11965092)
25 or 50 ml FBS (GE Healthcare, SH30396.03HI)
5 ml 200 mM L-glutamine (Thermo Fisher, 25030081)
5 ml 10,000 U/ml penicillin/streptomycin (Thermo Fisher, 15140122)
Store up to 1 month at 4°C

Iodixanol solutions, 15%, 25%, and 40%

15% iodixanol (35 ml):
26.2 ml PBS-MKN (see recipe)
8.8 ml Opti-Prep (60% iodixanol, Sigma-Aldrich, D1556)

25% iodixanol (30 ml):
17.5 ml PBS-MK (see recipe)
12.5 ml Opti-Prep

40% iodixanol (25 ml):
8.2 ml PBS-MK (see recipe)
16.8 ml Opti-Prep
Mix all solutions well by vortexing
Store up to 1 month at 4°C

PBS-MK

500 ml PBS, pH 7.4 (Thermo Fisher, 10010023)
101.66 mg $MgCl_2$ hexahydrate (Sigma, M9272; final 1 mM)
93.2 mg KCl (EMD, PX1405.1; final 2.5 mM)
Filter through a 0.22-µm membrane to sterilize
Store up to 1 month at 4°C

PBS-MKN

500 ml PBS, pH 7.4 (Thermo Fisher, 10010023)
37.99 g NaCl (Fisher Chemical, S271-10; final 1.3 M)
101.65 mg $MgCl_2$ hexahydrate (Sigma, M9272; final 1 mM)
93.2 mg KCl (EMD, PX1405.1; final 2.5 mM)
Filter through a 0.22-µm membrane to sterilize
Store up to 1 month at 4°C

PEI Max solution, 1 mg/ml

Dissolve 100 mg PEI Max powder (Polysciences, 24765-1) in 100 ml ultrapure distilled H_2O (Invitrogen, 10977015). Adjust pH to 7.1 with 5 N NaOH (Sigma, 221465). Filter through a 0.22-µm membrane to sterilize. Prepare 1-ml aliquots and store up to 6 months at 4°C.

Vector stock buffer

500 ml ultrapure distilled H_2O (Invitrogen, 10977015)
5.26 g NaCl (Fisher Chemical, S271-10; final 180 mM)
0.89 g $Na_2HPO_4 \cdot 2H_2O$ (Millipore, 567550; final 10 mM)
50 µl 100× Pluronic F68 (Thermo Fisher, 24040032; final 0.001%)
Adjust pH to 7.4 with 1 M HCl (Ricca, 3780-5PT)
Filter through a 0.22-µm filter to sterilize
Store up to 1 month at 4°C or aliquot and store up to 1 year at –80°C

COMMENTARY

Background Information

Over the past several decades, methods have been developed and optimized to increase recombinant AAV vector yield and purity. Initially, AAV was produced in mammalian cells that were transfected with two vector plasmids: one containing the transgene flanked by viral ITRs, the other helper plasmid containing the AAV coding genes *rep* and *cap in trans,* followed by adenovirus (Ad) infection (Hermonat & Muzyczka, 1984). However, this method could not avoid contamination from the Ad virus. After identifying the Ad genes *E1a*, *E1b*, *E2a*, *E4*, and *VA* required for AAV helper function, an Ad helper plasmid was developed containing the *E2a*, *E4*, and *VA* genes. HEK 293 cells, which endogenously contain *E1a* and *E1b,* were chosen to package cells (Xiao, Li, & Samulski, 1998). To improve vector yield, HEK 293 cells were adapted to grow in suspension, thus providing a platform for large-scale manufacturing of AAV vector (Hildinger, Baldi, Stettler, & Wurm, 2007). Some stable cell lines containing integrated copies of *rep* and *cap* have also been generated to facilitate AAV production (Chadeuf & Salvetti, 2010). Mammalian cell systems have been predominant in AAV production, although insect cells, such as Sf9 cells, have also been developed for AAV production by integrating AAV genes under the control of a baculovirus-specific promoter (Aslanidi, Lamb, & Zolotukhin, 2009).

The most popular and straightforward method for producing AAV is transient transfection of adherent HEK 293 cells. The advantage of transient transfection is that it permits the manufacturing of different serotypes of rAAV by changing only the *cap* genes in the pRepCap plasmid. Calcium phosphate precipitation of plasmid DNA has been the most widely used transfection method for rAAV production (Xiao et al., 1998). However, calcium phosphate transfection is sensitive, as slight variations in pH will affect transfection efficiency and AAV production. PEI is also

used for transfection (Hildinger et al., 2007). It is a highly charged cationic polymer that readily binds with highly anionic plasmid DNA. PEI has several advantages, including that it is well tolerated by HEK 293 cells, it efficiently transfects HEK 293 cells, it is inexpensive compared to commercially available lipid transfection reagents, and it is able to give reproducible results. PEI Max is a fully hydrolyzed, highly water soluble hydrochloride salt of PEI. PEI Max is easier to handle and has higher water solubility compared to PEI. Transfection efficiency with PEI Max is also higher than that of PEI when tested in our laboratory (data not shown).

Conventional rAAV purification involves multiple rounds of overnight cesium chloride gradient centrifugation (Gray et al., 2011). This purification method produces reasonably pure vector when performed correctly. However, it is time-consuming and prolonged exposure to CsCl may affect the potency of the AAV vector (Zolotukhin et al., 1999). More recently, an iodixanol gradient has been developed for rAAV purification. This method is achieved after one hour of centrifugation and has low toxicity (Zolotukhin et al., 1999). Furthermore, ion-exchange chromatography and affinity chromatography have been developed for large-scale AAV production (Auricchio, O'Connor, Hildinger, & Wilson, 2001; O'Riordan, Lachapelle, Vincent, & Wadsworth, 2000).

Critical Parameters

The final yield of AAV is dependent on transfection efficiency, which correlates to the state of cell health. Optimal transfection efficiency is achieved by passaging the cells twice per week to keep them in exponential growth conditions and splitting the cells prior to reaching 90% confluence. It is recommended to keep cells lower than passage 25 and to transfect the cells at 80% confluence. Investigators should test the PEI Max transfection solutions for their efficiencies before undertaking AAV production. Pilot experiments can be done by transfecting with pTransgene expressing a fluorescent gene into HEK 293 cell in one 145-mm plate. Expression is monitored by fluorescence microscopy; over 70% of cells should show evident expression after 24 hr.

Troubleshooting

A common problem with AAV production is low yield. Low yield can be caused by low transfection efficiency (referred to in Critical Parameters) or loss of integrity in the ITR. ITR integrity can be tested by restriction digest with enzymes such as SmaI, MscI, or XmaI.

Anticipated Results

In skilled hands, it should be possible to obtain 10^{12}-10^{13} genomic viral particles from ten 145-mm plates.

Time Considerations

rAAV production: Seeding the cells takes ~1 hr. Transfection takes 30 min. Harvesting cells takes 1 hr. Processing media and cells takes 2 hr.

rAAV purification: Preparing the solution takes 30 min. Loading the gradient and sample takes 30-60 min depending on the prep volume. Fraction or gradient collection takes 15 to 30 min. Diafiltration and concentration take 2 hr.

rAAV Quantification: Preparing the plasmid takes 30 min. DNase treatment takes 45 min. Q-PCR takes 15 min for setup and 2 hr to run the program.

Conflict of Interest

B.L.D. is a founder of Spark Therapeutics, Inc. and Talee Bio, Inc., and is on the scientific advisory board of Sarepta Therapeutics, Homology Medicines, Prevail Therapeutics, and Intellia Therapeutics. The content of this submission was not financially supported or reviewed by any of the aforementioned entities.

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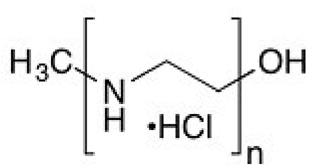

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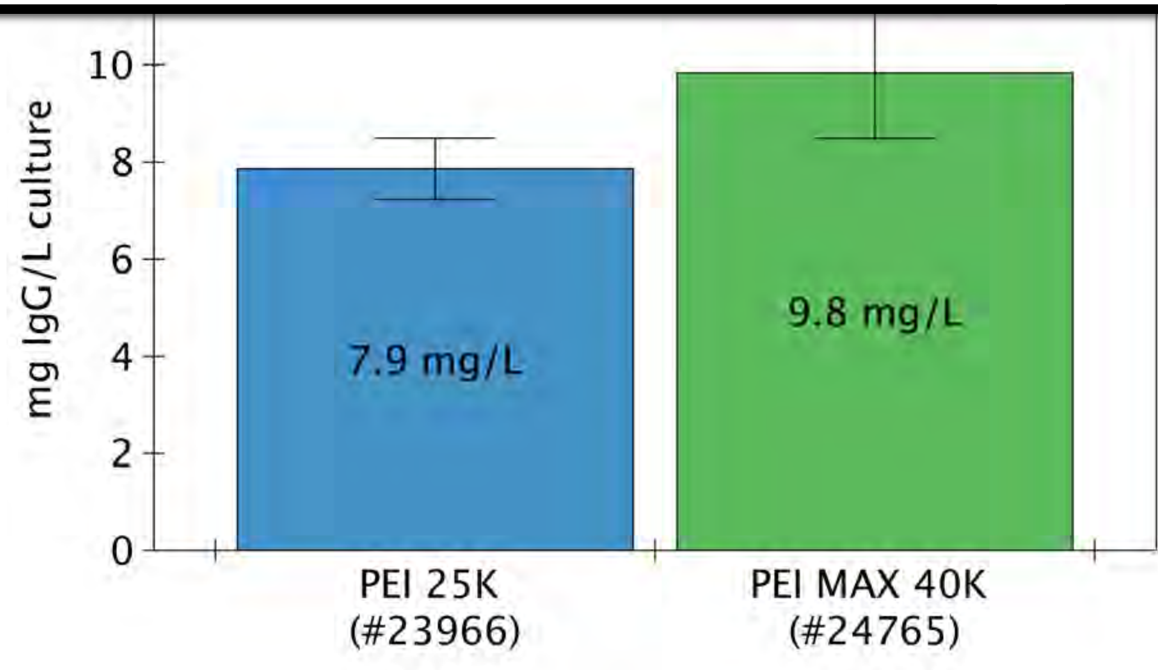

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Baranyi, L. et al. Rapid Generation of Stable Cell Lines Expressing High Levels of Erythropoietin, Factor VIII, and an Antihuman CD20 Antibody Using Lentiviral Vectors. Human Gene Therapy Methods 24, 214–227 (2013). doi:10.1089/hgtb.2013.002

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26008-50	50 mL	$495.00	0

Add to Cart

Bulk Quote

DESCRIPTION

Transporter 5™ is a premium ready-to-use transfection reagent prepared from our popular linear polyethylenimine MAX (PEI MAX 1 mg/mL). Transporter 5™ effectively transfects mammalian and insect cells, especially HEK-293, CHO, and Sf9.

We understand every transfection is a major investment, so we designed Transporter 5™ to be a reliable reagent in any process.

The quality of Transporter 5™ begins with our production process. Our special production method for PEI creates pure polymer with no batch-to-batch molecular weight variation. Transporter 5™ combines our consistent PEI with the highest quality USP/NF reagents in sterile, single-use equipment. While standard methods use 0.2µm sterile-filters, we use 0.1µm to completely eliminate mycoplasma risk.

Our Quality Control ensures Transporter 5™ is the most reliable reagent available. We developed our own scientifically-sound, quantitative performance assay for Transporter 5™. Most commercial performance assays are based on qualitative assays like SEAP and GFP. These assays cannot ensure the high titers most scientists

Appearance: Liquid
Reference(s):

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Wulhfard, S., Baldi, L., Hacker, D. L. & Wurm, F. Valproic acid enhances recombinant mRNA and protein levels in transiently transfected Chinese hamster ovary cells. *Journal of Biotechnology 148*, 128–132 (2010). doi:10.1016/j.jbiotec.2010.05.003

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Expired Patent: US6013240; Nucleic Acid Containing Composition, Preparation, and Uses of the Same

Hazards: Harmless, use normal precautions
Handling: Gloves & chemical googles
Storage: Store at 4 degree C; do not freeze. Shipped at room temperature (RT).

MSDS / TECHNICAL DATA SHEETS / PRODUCT LITERATURE

MSDS MSDS
DATA Data Sheet #1024
LIT Flyer
LIT BSE/TSE Statement

RELATED PRODUCTS

Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K)

PEI MAX - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000)

US Headquarters
Polysciences, Inc.
400 Valley Road
Warrington, PA 18976

1 (800) 523-2575 or (215) 343-6484
fax: 1 (800) 343-3291 or (215) 343-0214
info@polysciences.com

European Sales and Distribution
Polysciences Europe GmbH
Badener Str. 13
69493 Hirschberg an der Bergstrasse, Germany

+(49) 6201 845 20 0
+(49) 6201 845 20 20 fax
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Asia Pacific Sales and Distribution
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Taipei, Taiwan 10595

(886) 2 8712 0600
(886) 2 8712 2677 fax
info@polysciences.tw

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Exhibit R

PRODUCTS

PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000)

View pricing for: **Americas/Asia**, Europe, Taiwan

Catalog No.	Packaging Size	Price	Quantity
24765-1	1 g	$1,035.00	0

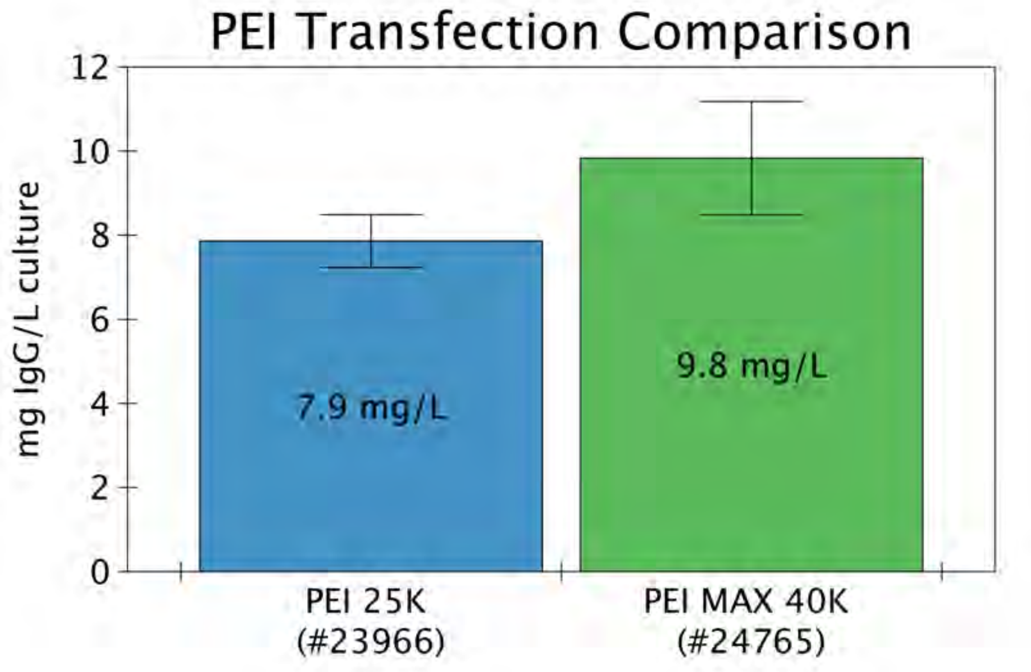
Add to Cart

Bulk Quote

DESCRIPTION

PEI MAX 40K (also known as PEI 22K in free base) is a powerful, trusted, and cost-effective transient transfection reagent. In HEK293 and CHO expression systems, PEI offers consistently high gene expression on a wide scale (96 well plates up to 100 L bioreactors). Each year, more researchers and companies turn to Polysciences PEI to gain an edge in their critical work. Relative to most other options, using PEI to prepare transfection reagents in-house can offer as much as a 40% reduction in total transfection costs.

PEI MAX 40K is easier to use and offers consistently higher titers than PEI 25K. PEI 25K transfection solutions typically take several hours to prepare, while PEI MAX 40K can be converted to a ready-to-use solution in under two hours. Additionally, PEI 25K contains 4-11% residual propionyl groups, which prevents the polymer backbone from strongly binding to DNA. PEI MAX 40K's fully depropionylated structure means each batch performs consistently higher.



Comparison of PEI 25K (#23966) and PEI MAX 40K (#24765).
Method: 10e6/mL HEK293 cells in 50 mL FS transfected with IgG64 plasmid pair. PEI:DNA 4:1. Samples taken 120 hpt. Quantified with Thermo Fisher #23310. N=4 each. Error bar = standard deviation.

A pre-made sterile-filtered solution of PEI MAX 40K is available as our Transporter 5™ Transfection Reagent.

CAS#: 49553-93-7
Molecular Weight: 40,000 (~22,000 free base)
Soluble In: Cold and room temperature water
Insoluble in: Common organic solvents (ethanol, acetone, tetrahydrofuran)
Appearance: White to off-white free flowing solid
Reference(s):

Baranyi, L. et al. Rapid Generation of Stable Cell Lines Expressing High Levels of Erythropoietin, Factor VIII, and an Antihuman CD20 Antibody Using Lentiviral Vectors. Human Gene Therapy Methods 24, 214–227 (2013). doi:10.1089/hgtb.2013.002

** Delafosse, L., Xu, P. & Durocher, Y. Comparative study of polyethylenimines for transient gene expression in mammalian HEK293 and CHO cells. Journal of Biotechnology 227, 103–111 (2016). doi:10.1016/j.jbiotec.2016.04.028

** Gutiérrez-Granados, S., Cervera, L., Segura, M. de las M., Wölfel, J. & Gòdia, F. Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells. Applied Microbiology and Biotechnology 100, 3935–3947 (2016). doi:10.1007/s00253-015-7213-x

Kobayashi, S., Yoshii, K., Hirano, M., Muto, M. & Kariwa, H. A novel reverse genetics system for production of infectious West Nile virus using homologous recombination in mammalian cells. *Journal of Virological Methods 240*, 14–20 (2017). doi:10.1016/j.jviromet.2016.11.006

** Longo, P. a, Kavran, J. M., Kim, M. & Leahy, D. J. Transient Mammalian Cell Transfection wtih Polyethylenimine (PEI). *Methods Enzymology 529*, 227–240 (2013). doi:10.1016/B978-0-12-418687-3.00018-5.

Mann JF, McKay PF, Arokiasamy S, Patel RK, Klein K, Shattock RJ. (2013). Pulmonary delivery of DNA vaccine constructs using deacylated PEI elicits immune responses and protects against viral challenge infection. J Control Release. 170(3):452-9.

Pinnapireddy, S. R., Duse, L., Strehlow, B., Schäfer, J., & Bakowsky, U. (2017). Composite liposome-PEI/nucleic acid lipopolyplexes for safe and efficient gene delivery and gene knockdown. *Colloids and Surfaces B: Biointerfaces*, *158*, 93-101.

** Stuible, M. et al. Optimization of a high-cell-density polyethylenimine transfection method for rapid protein production in CHO-EBNA1 cells. Journal of Biotechnology 281, 39–47 (2018). doi:10.1016/j.jbiotec.2018.06.307

Thomas M, Lu JJ, Ge Q, Zhang C, Chen J, Klibanov AM. (2005). Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proc Natl Acad Sci U S A.* 102(16):5679-84.

** Contains particularly useful information.

Hazards: Irritant
Handling: Glove, chemical goggles & fume hood
Storage: Store at room temperature

MSDS / TECHNICAL DATA SHEETS / PRODUCT LITERATURE

MSDS Material Safety Datasheet 24765 (PDF)

DATA FT-IR Spectrum

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Transporter 5™ Transfection Reagent

View pricing for: **Americas/Asia**, Europe, Taiwan

Catalog No.	Packaging Size	Price	Quantity
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Appearance: Liquid

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Choosakoonkriang, S., Lobo, B. A., Koe, G. S., Koe, J. G. and Middaugh, C. R. (2003), Biophysical characterization of PEI/DNA complexes. *J. Pharm. Sci., 92*: 1710–1722. doi: 10.1002/jps.10437

Expired Patent: US6013240; Nucleic Acid Containing Composition, Preparation, and Uses of the Same

Hazards: Harmless, use normal precautions

Handling: Gloves & chemical goggles

Storage: Store at 4°C; do not freeze. Shipped at room temperature (RT).

MSDS / TECHNICAL DATA SHEETS / PRODUCT LITERATURE

MSDS MSDS

DATA Data Sheet #1024

LIT PEI Transfection Reagents

RELATED PRODUCTS

Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K™)

MAXgene™ GMP Transfection Reagent

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info@polysciences.com

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69493 Hirschberg an der Bergstrasse
Germany

Asia Pacific Sales and Distribution

Polysciences Asia Pacific, Inc.
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(886) 2 8712 2677 fax

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Exhibit S

Polysciences, Inc.
Chemistry beyond the ordinary
400 Valley Road • Warrington, PA 18976

CERTIFICATE OF ANALYSIS

Product Name: POLYETHYLENEIMINE 'MAX'(40 000M.W. LINEAR

Catalog #: 24765

Lot Number: 706510

Parameter	Specifications	Results	
Transfection Performance Testing (IgG Expression in HEK293)	≥ 7.0	10.8	mG/L
Residual Propionyl Group %	< 3.0	0.2	%
Inherent Viscosity (25°C, 1% aqueous solution)	0.45 - 0.90	0.74	dL/g
FT-IR	conforms to standard	Pass	

Date: Tuesday, August 01, 2017

www.polysciences.com
Email: info@polysciences.com

Exhibit T

Transporter 5™ Transfection Reagent

The Solution For Protein Expression

Transporter 5™ is a ready-to-use transient transfection reagent of linear polyethylenimine (PEI).

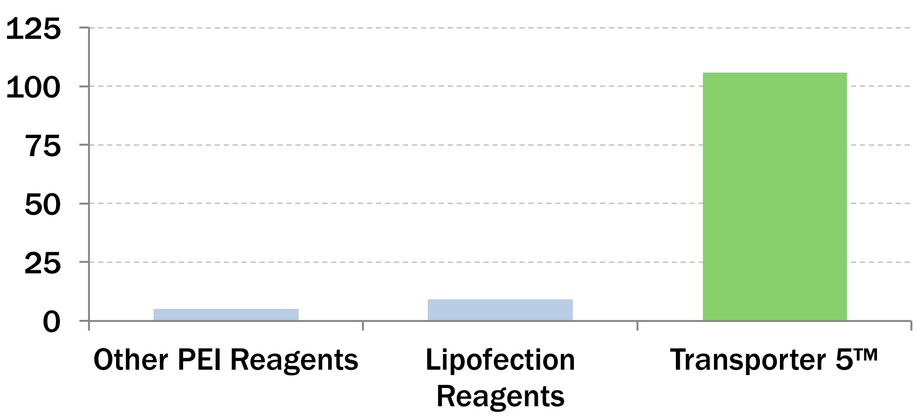
Applications

- High-throughput screening (HTS) of protein
- High-volume protein and lentivirus production

Value

Transporter 5™ offers industry-leading value for transfection reagents.

Transfections per $



Graph based on 96-well transfections. The best value for each product group was used.

Part Number
26008

Reagent
Linear Polyethylenimine (PEI)

Concentration
1 mg/mL (PEI HCl)
12.6 mM (amines)

Expression Time
4 to 8 days

PEI : DNA Ratio (w : w)
(3 to 7) : 1

Cell Lines
CHO
HEK293

Specification
IgG Expr (120 h.) ≥ 6.5 mg/L

Shelf Life
2 years

Additional Info
www.polysciences.com/t5

America, Asia, Oceania
info@polysciences.com
www.polysciences.com
(P) 1 (800) 523-2575
(F) 1 (800) 343-3291

Europe
info@polysciences.de
www.polysciences.de
(P) +(49) 6201 845 20 0
(F) +(49) 6201 845 20 20

Taiwan
info@polysciences.tw
www.polysciences.tw
(P) (886) 2 8712 0600
(F) (866) 2 8712 2677

Polysciences, Inc.

Quality

Each batch of Transporter 5™ is prepared by experienced chemists in our ISO 9001 facility in Warrington, PA. We use disposable, sterile containers for the entire manufacturing and packaging process. 0.1 micron sterile-filters prior to packaging minimize the risk of bacterial or mycoplasma contamination.

Transporter 5™ is checked for transfection efficiency in HEK293 cell lines using a Human IgG expression assay.

Ordering Information

Up-to-date pricing and availability information is always available at **www.polysciences.com/t5**.

SKU	Size	(96 Well) Transfections	Culture Volume	DNA
26008-1	1 mL	2,000	150 mL – 300 mL	150 µg – 300 µg
26008-5	5 mL	10,000	750 mL – 1,500 mL	750 µg – 1,500 µg
26008-50	50 mL	100,000	7.5 L – 15.0 L	7.5 mg – 15.0 mg

Need Other Sizes?

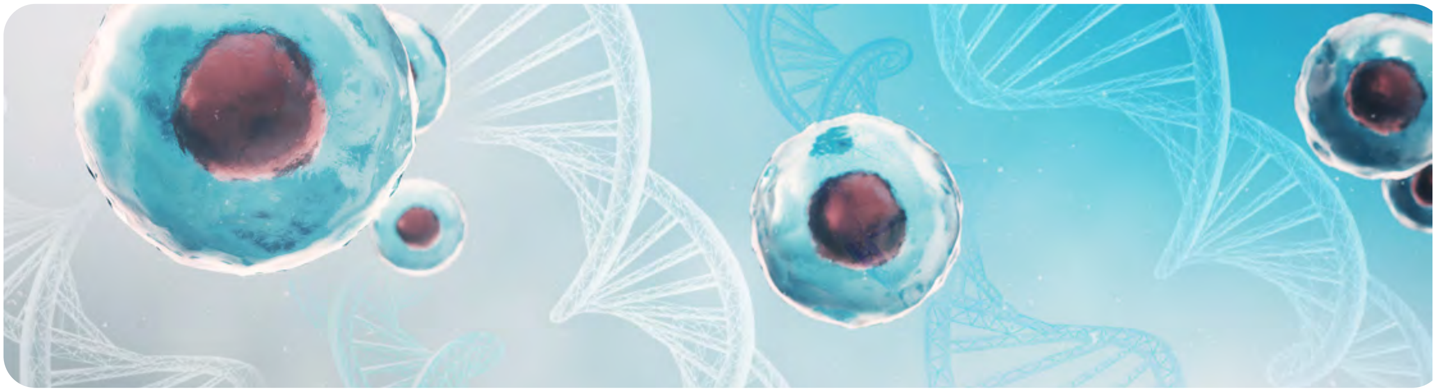
We are happy to help. Our flexible production and packaging process is designed to meet requests up to 20L with any packaging arrangement. Please send your inquiry to **bulk@polysciences.com** and we will get back to you promptly.

Exhibit U

MAXgene™ GMP

GMP Grade Transfection PEI

Polysciences
Chemistry beyond the ordinary



Our premier transfection reagent for accelerated clinical therapies

MAXgene™ GMP Transfection Reagent is a cGMP transfection reagent for the development and manufacturing of viral vectors for cell-and gene-based therapies. It is an ideal reagent in HEK293 and CHO systems for the manufacture of AAVs, LVs and recombinant proteins. MAXgene™ GMP capitalizes on the efficiency and scalability of Polysciences' PEI MAX while adding the validation process and regulatory components necessary for moving into clinical and commercial manufacturing. It is manufactured in accordance with cGMP under an ISO 13485 Quality Management System.

HIGHLIGHTS

- Compatible with different virus production platforms
- High transfection efficiency
- Reliable and scalable performance
- cGMP & ISO 13485
- Validated manufacturing processes
- Fully synthetic, animal-origin-free
- Cost-effective

ATTRIBUTES

Appearance	Clear liquid	
Identity	FT-IR†	
Molecular Weight	GPC conforms†	
Impurity Profile	Residual propionyl†, Residual solvents†	
Storage:	Store at 2-8° degrees C; do not freeze.	
pH	6.3-6.7	
Osmolality	Complies	
Performance - IgG Expression (HEK293) Transfection Efficiency (HEK293) Viability (HEK293)	Complies	
USP 71 Sterility Testing	Complies	
USP 63 Mycoplasma	Negative	
USP 232 Heavy Metals	Element	Limit ppm
	Cd	8
	Pb	25
	As	60
	Hg	12
	Ir	40
	Os	40
	Pd	40
	Pt	40
	Rh	40
	Ru	40
	Cr	4400
	Mo	6000
	Ni	80
	V	40
	Cu	1200

† Analysis performed on dry precursor (data represents liquid form)

PEI for Transfection (Clinical Grade)

Cat. #	Description	Size
26406-1	MAXgene™ GMP	1 L tamper free bottle
26435	MAXgene™ GMP Powder	1, 10, 50g

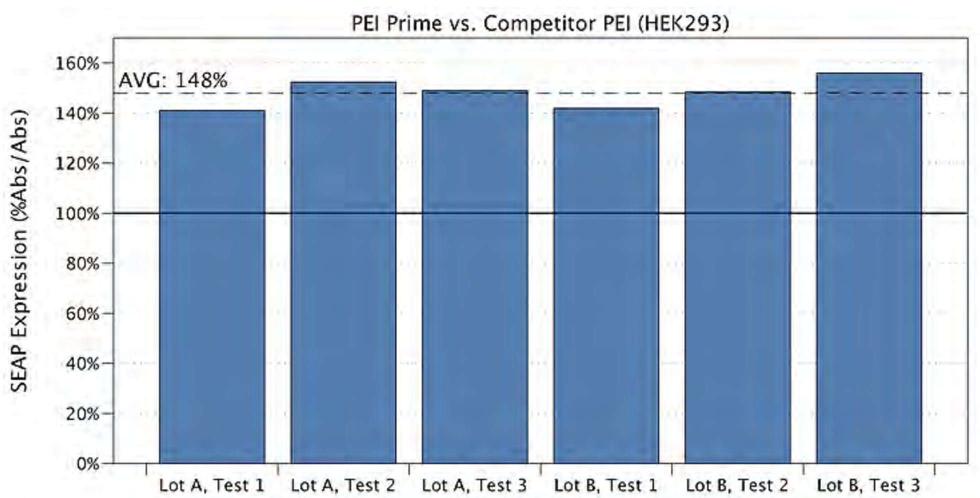
Order today at **Polysciences.com**

Exhibit V

Jul 20

PEI Prime™ Performance Data (HEK293)

PEI Prime™ is an exceptional, high-performance transfection reagent for HEK293 suspension cultures. Transient gene expression (TGE) using PEI Prime™ shows expression levels significantly higher (48%) than the leading competitor PEI.



Method: 20 mL cultures containing HEK293 suspensions were transfected with a CMV-SEAP plasmid at optimized PEI/DNA ratios using either PEI Prime™ (3:1 here) or leading competitor PEI. SEAP expression levels were quantified 5 days post-transfection using phosphatase reporter dye and UV/Vis. Values are reported as percentage of leading competitor PEI.

4 views

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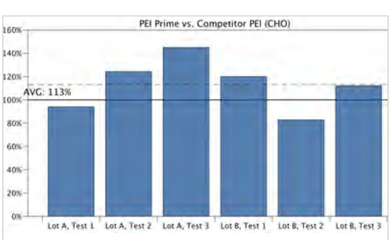

PEI Prime™ Performance Data (CHO)

3

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Exhibit W

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Transporter 5™ Transfection Reagent

View pricing for: **Americas/Asia**, Europe, Taiwan

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Expired Patent: US6013240; Nucleic Acid Containing Composition, Preparation, and Uses of the Same

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astraldiagnostics incorporated
Ethos Biosciences

Note	*Some of the stock solutions come with the pH indicator phenol red. This supplement does not affect the application and might be useful if the researcher wishes to visualize any pH changes that can occur in the solutions over time. In the case of non-CO_2 incubators (e.g., when scaling-up the production of adherent cells in roller bottles), HEPES-buffered media can be used to keep the pH stable.*
Note	*Catalog numbers are from the US website of Invitrogen and may differ on other local websites.*

3.1. Solutions & buffers

PEI 'Max'		
Dissolve 1 g PEI 'Max' in 900 ml distilled water. Adjust the pH to 7.0 with 1 N NaOH. Add distilled water to 1 l		
Note: Stable at least 9 months at 4 °C		
Make smaller volumes depending on how much is needed. PEI 'Max' cannot be frozen!		
FreeStyle™ 293 'Completed'		
Component	**Stock**	**Amount**
FreeStyle™ 293 medium		1 l
FBS	100%	10 ml
L-Glutamine	200 mM	10 ml
DMEM:F12, 5% FBS		
Add 50 ml FBS to 1 l of DMEM:F12		
Alpha MEM, 5% FBS		
Add 50 ml FBS to 1 l of Alpha MEM		
Hybridoma SFM, 1% FBS		
Add 10 ml FBS to 1 l of Hybridoma SFM		

4. PROTOCOL

4.1. Preparation

Before transfection, sterile high-quality DNA must be prepared. The vector containing the appropriate expression promoter (see Molecular Cloning) and the gene of interest should be transformed into a recA- strain of *E. coli* (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation) and then the plasmid DNA isolated (see Isolation of plasmid DNA from bacteria). Commercially available, endotoxin-free kits for large-scale plasmid DNA isolation produce

L1 RNPs purified by affinity capture provide the purest, most active L1 elements reported to date and are excellent starting material for assay by mass spectrometry, LEAP, and qRT-PCR [6]. We provide several of our working protocols for L1 sample preparation and analysis.

2 Materials

2.1 Suspension Cell Culture

1. Humidified CO_2-controlled tissue culture incubator.
2. Orbital shaker platform at 130 rpm fitted with racks.
3. 20 mm 40-place test tube racks.
4. Diagonal cutting pliers, flat wood file, bandsaw (for modifying racks).
5. Corning Pyrex 1 L glass bottles.
6. 7×® Cleaning solution (Bellco Glass).
7. Hybridoma SFM medium (Life Technologies).
8. Freestyle 293 Medium (Life Technologies).
9. Opti-MEM Reduced Serum Medium.
10. TrypLE Express (Life Technologies).
11. Certified tetracycline-Free FBS (Tet-free FBS).
12. DMEM medium.
13. Phosphate-buffered saline (PBS).
14. PEI Max (MW 40,000), Polysciences: Two grams is enough to transfect >600 L.
    (a) To prepare working 1 mg/mL PEI Max solution:
    (b) Dissolve 100 mg PEI Max in 90 mL ddH_2O.
    (c) Adjust pH to 7.0 using 1 M NaOH.
    (d) Adjust volume to 100 mL, filter sterilize, and store at 4 °C.
    (e) *NEVER FREEZE PEI working stock*. Working stocks can be used for up to 6 months if stored at 4 °C.

2.2 Cell Harvest

15. Large-volume floor centrifuge with appropriate rotor (e.g., 4 × 1 L, 6 × 500 mL).
16. 16ga needles.
17. Luer-lock syringes, 5 mL, 10 mL, or 30 mL.
18. Luer-lock syringe end caps (BioRad).
19. Liquid nitrogen and Dewar flask.
20. Gloves for handling liquid nitrogen.
21. Small Styrofoam box.